Studies on amniotic fluid cells in culture with emphasis on the antenatal diagnosis of genetic disease.

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Summary

SUMMARY

In view of the increasing use of amniotic fluid for the antenatal diagnosis of fetal abnormality there was a need for information about the biology of amniotic fluid cells in tissue culture. The behaviour of these cells in primary and long term culture has been documented, cytogenetic studies have been carried out to assess the reliability of this technique and the characteristics of some lysosomal enzymes in normal amniotic fluid cell strains have been studied. Two new findings were made and investigated during this study, the increase in amniotic fluid macrophages in anencephaly and the ability of urine cells to proliferate in tissue culture.

A high degree of success was achieved in initiating primary cultures from amniotic fluid cells. Success in culture was found to be independent of the source of the amniotic fluid with the exception that fluids collected at induction of labour at term could not be reliably cultured. Fluids from Rhesus iso-immunised pregnancies grew well in culture and their growth characteristics were unrelated to the severity of this disease. Studies on the methods of primary culture indicated that the maintenance schedule of the cultures was not relevant to the rate of cell growth and that amniotic fluid itself was not as good as conventional culture media for initiating cell growth in primary culture. The morphology of the cells in amniotic fluid cultures was studied.

The behaviour of amniotic fluid cell strains

throughout serial culture to the stage of senescence was documented. Changes in cell morphology with time in culture were monitored. It was found that faster growing cell strains survived a greater number of sub-cultures than did the slower growing ones. Some cytogenetic studies on the longest surviving cell strains demonstrated that karyotype stability was a feature of these cell strains except in the senescent phases when chromosome abnormalities similar to those seen in senescent fibroblast-like cell strains were seen. The time required to produce enough cells for biochemical studies of cultured amniotic fluid was measured and the problem of achieving this in sufficient time is discussed.

Cytogenetic studies on amniotic fluid from pregnancies in which the fetus was considered to be at risk for a chromosome abnormality were carried out and the antenatal diagnosis of trisomy 18 was documented. This study indicated that antenatal chromosome analysis is a simple, rapid and reliable means of establishing the fetal karyotype and would be applicable to selective population screening to reduce the incidence of chromosome abnormality at birth.

It was found that amniotic fluid from anencephalic pregnancies had greatly increased numbers of fetal macrophages and the possibility of using this finding as a test for the antenatal diagnosis of anencephaly has been explored. Amniotic fluid from two spina bifida amniotic fluids had slightly increased numbers of macrophages. Rhesus iso-immunised amniotic fluids had

slightly increased macrophage counts and this may be associated with the increase in placental Hofbauer cells in this condition.

The levels of 10 lysosomal enzymes in cultured amniotic fluid cells were studied. The normal range of these enzyme levels was established. It was found that there were great fluctuations in the levels of these enzymes within single cell strains assayed at different stages of culture. Possible explanations for these fluctuations were sought by studying the effects of pH of the culture medium, type of culture medium and serum concentration used on the levels of the enzymes. Thirteen cell strains were produced from one sample of amniotic fluid and assayed to study the enzyme variation due to the culture methods used. Pregnancies at risk for maple-syrup-urine disease, metachromatic leukodystrophy and Sandhoff's disease were monitored to assess the status of the fetus.

It was found that cells from the urine of newborn infants would grow in tissue culture. The behaviour and morphology of these cells were markedly similar to those cultured from amniotic fluid. The lysosomal enzyme biochemistry of urine cell strains was more similar to that of amniotic fluid cell strains than that of connective tissue derived fibroblast-like cell strains. The hypothesis that the cells which grow from amniotic fluid are derived, at least in part, from the fetal urinary tract is discussed.

An appendix contains 14 papers published or accepted

for publication during this study. These have made significant contributions in documenting the behaviour of amniotic fluid cell strains, in the discovery of the ability of urine cells to grow in culture and in the finding of fetal macrophages in anencephalic amniotic fluid. The papers on lysosomal enzyme studies of cultured amniotic fluid cells constitute the bulk of published work in this field.

Chapter 1

Introduction and Historical Background

INTRODUCTION

The prediction of disease in the human fetus sufficiently early to allow for the subsequent termination of affected pregnancies was little more than a eugenicist's dream until 1955. In that year, several groups independently showed that sex chromatin, and hence the sex of the fetus, could be determined by examination of the cells present in amniotic fluid. This finding was the first step in the development of methods for detecting a number of diseases in the fetus in utero.

In 1963 it was reported that amniotic fluid cells could be cultivated in tissue culture. Following this achievement two areas of study using cultured cells were opened up, namely cytogenetics and human biochemical genetics. For the reliable antenatal diagnosis of genetic disorders cultured amniotic fluid cells are almost always necessary. Hence it is essential that the behaviour and properties of these cells in tissue culture be well known.

The aim of this study was to achieve a deeper understanding of the biology of amniotic fluid cells in tissue culture, using a number of projects each directed towards this general goal but each with its own specific aim:

1. To study amniotic fluid cells in tissue culture and identify factors affecting growth in primary culture.

2. To study the cell strains derived from primary amniotic fluid cultures and to see how they relate to

other human diploid cell strains.

3. To perfect methods for obtaining chromosome preparations from amniotic fluid suitable for the diagnosis of fetal chromosome abnormalities before mid pregnancy. For this purpose a diagnostic service was established for karyotyping amniotic fluids referred from genetic and obstetric clinics.

4. Towards the end of this work it was found that amniotic fluid from two anencephalic pregnancies contained large numbers of macrophages. Further studies have been carried out on the macrophage content of normal and abnormal amniotic fluids.

5. To study a number of lysosomal enzymes in cultured amniotic fluid cell strains and investigate the variables in tissue culture which may affect the activities of the enzymes. This investigation would provide a knowledge of these particular enzymes, all except one of which are involved in inborn errors of metabolism.

6. Early in this work it was discovered that the cells from urine of neonates could be grown in tissue culture. As a result of this finding urine cells were cultured and compared with amniotic fluid cells in an attempt to ascertain whether fetal urine contributes significantly to the cellular content of amniotic fluid. The feasibility of using cultured urine cells, rather than fibroblast-like cells cultured from skin biopsy, as a model for the biochemical study of inborn errors of metabolism was investigated. The use of urine cell strains may be more suitable for such studies which might

have to be extrapolated to amniotic fluid cells for antenatal diagnosis.

This thesis records the observations made on amniotic fluid cells in tissue culture and relates these observations to antenatal diagnosis of genetic disease. In summary it records a study of the anatomy and physiology of amniotic fluid cells in tissue culture - essential knowledge for the study of their pathology.

HISTORICAL BACKGROUND

There has long been interest in the amniotic fluid, its origins and composition. Writing in the seventeenth century Portal (quoted by Williams, 1930) supported the view that amniotic fluid was composed of fetal urine. The first study of the composition of amniotic fluid to be reported in the English language was made by Rees in 1838. He analysed four samples of fluid, all from pregnancies of about $7\frac{1}{2}$ months gestation, for water, albumen and organic and inorganic matter in aqueous and alcoholic extracts. He concluded: "Whether or not the liquor amnii varies in concentration at different periods of utero-gestation remains to be shewn: it is certainly pretty constant at $7\frac{1}{2}$ months." The first major work on the composition of amniotic fluid was made by Prochownick in 1877. In a comprehensive paper he reported quantitative and qualitative analyses of protein, fats, other organic materials, urea, salts and total inorganic matter in a series of amniotic fluids ranging in gestational age from 6 weeks to term. He was of the opinion that amniotic fluid was a product of fetal metabolism. The next major work on the composition of amniotic fluid was that of Uyeno (1919) who studied a number of components including pH, inorganic ions, sugars and other organic compounds as well as its physical properties. In 1927 Taussig reviewed the composition of amniotic fluid and stated that there was "..... no reasonable doubt that amniotic fluid is secreted by the amnion". He did, however, allow that there may have been

some admixture of fetal urine shortly before labour.

Up to the mid 1930's a few papers appeared on the composition of amniotic fluid, some compared the levels of various constituents in the amniotic fluid and maternal blood (Makepeace et al., 1931; Cantarow et al., 1933; Shrewsbury, 1933). There was little of importance published on the composition of amniotic fluid from this time until 1950.

The first application of the study of amniotic fluid was in the management of fetuses suffering from Rhesus iso-immunisation. Brevis (1950, 1952) studied the composition of amniotic fluid in this condition. The clinical application of amniotic fluid bile pigment measurements in the management of pregnancies complicated by Rhesus iso-immunisation was developed by Liley (1961). The use of amniotic fluid for this purpose has become so universal that Scrimgeour (1973) was able to review 21,000 amniocenteses from series published between 1952 and 1970. Parrish et al. (1958) discussed the obstetric problems involved in collecting amniotic fluid prior to delivery and introduced the term "transabdominal amniocentesis" for the procedure.

The mid 1960's saw an explosion in the number of papers published on the composition of amniotic fluid and Bonsnes (1966) reviewed the findings up to this stage. Much of the work since this time has been aimed at finding a component which will correlate with fetal respiratory maturity and hence improve obstetric care. One such parameter is the lecithin/sphygomyelin ratio (Whitfield

et al. 1972).

Some amniotic fluid biochemistry has however been aimed at measuring the levels of components which could be expected to alter if the fetus was suffering from a particular inborn error of metabolism. Fuchs (1960) suggested this as a possible approach to the antenatal diagnosis of inborn errors of metabolism. Compounds measured in this regard include enzymes (Bonsnes, 1966; Butterworth et al., 1972, 1974; Fluharty et al., 1973; Sutcliffe et al., 1972), amino acids (Emery et al., 1970 ; Cockburn et al., 1970), electrolytes (Emery, 1970) and steroid hormones (Jeffcoate et al., 1965).

The use of studies on amniotic fluid alone as the basis for antenatal diagnosis of inborn errors of metabolism has been strongly criticised for most inborn errors with a possible exception being methymalonic acidaemia (Morrow et al., 1970). There is one case report which suggests that enzyme studies on amniotic fluid should be diagnostic in Sandhoff's disease (Desnick et al., 1973). The most promising approach to the antenatal diagnosis of fetal abnormality by a study of amniotic fluid has been that of Brock and Sutcliffe (1972). They showed elevated levels of α-fetoprotein in amniotic fluid from pregnancies where the fetus had anencephaly.

Interest in the cells in amniotic fluid was first shown by Daniel (1904) who studied the cytology of 38 amniotic fluids obtained by rupture of the membranes at delivery. In the mid 1950's there were at least six

independent reports (see Emery, 1970 for references) of the finding of sex chromatin in amniotic fluid cells. The ability to sex the fetus before birth was thus finally achieved after centuries of speculation based on a host of folk recipes going back to those used by the ancient Greeks and Egyptians (Blakely, 1937). Cederqvist and Fuchs (1970) have reviewed the historical development of antenatal sex determination.

The ability to sex the fetus was first used genetically by Riis and Fuchs (1960) to manage the pregnancies of two women who were carriers of haemophilia. Few laboratories have achieved 100% success in sexing amniotic fluid using either sex chromatin or Y-fluorescence methods (see Nelson, 1973a for analysis of success rates) and it is generally recommended that fetal sex be determined from the chromosomes (e.g., Nadler and Gerbie, 1971).

In 1960 Fuchs, and Fuchs and Riis, in two articles in the same copy of Nordisk Medicin suggested that it might be possible to culture amniotic fluid cells and detect chromosome abnormalities in the fetus. This was a truly remarkable suggestion coming in the same year as the classic paper of Moorhead et al. on the technique for making chromosome preparations and at a time when tissue culture was largely the preserve of highly specialised units. In addition, at this time the extent of chromosome abnormalities was not known as it was only a year since the description of the first chromosome abnormalities in man (see Sutherland, 1970

for references). Three years later Fuchs and Philip (1963) reported that they had successfully cultured the cells from 2 out of 3 samples of amniotic fluid but that their experiments had to be discontinued before chromosome studies could be attempted.

Another two years elapsed before the culture of amniotic fluid cells was documented, the prediction of Fuchs (1960) and Fuchs and Riis (1960) was fulfilled and the work of Fuchs and Philip (1963) confirmed. There is controversy about who was the first to grow amniotic fluid cells in tissue culture, the work of Fuchs and Philip (1963) apparently having been lost in the literature. The first full report was that of Steele and Breg (1966) who quoted a personal communication from Klinger (1965), the one generally credited with being the first to grow these cells. Jacobson has claimed to be the first to culture these cells (Jacobson, 1965 quoted by Jacobson and Barter, 1967). However examination of the claim suggests that misquotation has occurred, the paper of Jacobson (1965) consists of three short paragraphs in a drug company newspaper in which it is stated that experiments were taking place in an effort to try and grow amniotic fluid cells; their successful culture had not been achieved. Macintyre (1971) claimed to be the co-discoverer of amniotic fluid culture with, but independently of, Klinger. There appears to be no report in the scientific literature to support Macintyre's claim.

Although there is one unconfirmed report of a fetal

karyotype having been produced from uncultured amniotic fluid cells (Hughes et al. 1971), cytogenetic studies of amniotic fluid have developed in parallel with the culture of amniotic fluid cells. The series of cases in which antenatal chromosome analysis has been performed for diagnostic purposes up to 1971 have been summarised by Sutherland (1972, appendix III). Since this time a number of series have been published, e.g., Milunsky et al. (1972); Therkelsen et al. (1972); Epstein et al. (1972).

The cells present in amniotic fluid do not appear to have been subjected to biochemical studies until about 1968. Dancis (1968) assayed uncultured cells for a number of enzymes but could detect only minimal amounts of valine transaminase and no activity of other enzymes tested. He readily detected valine transaminase in cultured amniotic fluid cells but again no other enzymes. Nadler and Gerbie (1969) presented qualitative results for a range of enzymes in both uncultured and cultured amniotic fluid cells. Sutcliffe and Brock (1971) assayed a number of enzymes in uncultured cells and concluded that this was inadequate material on which to base diagnoses. Berman et al. (1969) suggested that if the activity of one enzyme was related to that of another, rather than to protein, reasonably consistent results could be obtained from such material. There is general agreement in the literature that uncultured amniotic fluid cells are not suitable material on which to base antenatal diagnosis of inborn errors of metabolism.

The first quantitative values for an enzyme in cultured amniotic fluid cells were given by Nadler (1968b) for glucose 6-phosphate dehydrogenase. Nadler (1968b) claimed to have assayed 8 enzymes in cultured amniotic fluid cells and stated that the levels did not change throughout pregnancy. Not a single assay value is given in support of this claim. It was claimed in the same paper that glucose 6-phosphate dehydrogenase decreased with time in culture when cells cultivated from amniotic fluid from 10 weeks female fetuses were assayed. This claim has never been verified and is strongly criticised by Brock (1973). Nadler (1968a) and Fujimoto et al. (1968) showed that the biochemical defect in some inborn errors of metabolism was present in cultured amniotic fluid cells. Since this time a number of enzymes have been measured in cultured amniotic fluid cells (see Brock, 1973, for references). However, these have usually been in small numbers of cell strains. Often antenatal diagnoses of inborn errors have led to a few "control" cell strains being assayed (e.g., Nadler and Messina, 1969).

There are no systematic studies in the literature of the behaviour of an enzyme or group of enzymes in cultured amniotic fluid cells. The normal values of enzyme activity quoted are based on studies of small numbers of cell strains. The levels of some enzymes in amniotic fluid cell strains have been compared with those in cultured skin fibroblast-like cells by Uhlendorf and Mudd (1968), Kabak and Leonard (1970) and others (see Brock,

1973 for references). This work emphasised the necessity of defining the "'normal characteristics' of cultivated amniotic fluid cells" (Nadler, 1969), and cautioned against the extrapolation of biochemical findings in fibroblast-like cell strains to cultured amniotic fluid cells.

Towards the end of this study of amniotic fluid cells in tissue culture, it was found that amniotic fluid from two anencephalic pregnancies contained large numbers of cells which appeared to be fetal macrophages (Sutherland et al., 1973b, appendix III). Since fetal macrophages had never been previously described in amniotic fluid the macrophage content of normal and abnormal amniotic fluids was studied.

The foregoing summarises the background against which this work commenced. There were large gaps in knowledge of the behaviour of amniotic fluid cells in tissue culture, both primary and long term. The biochemistry of amniotic fluid cell strains had not been studied systematically for even a single component. The antenatal diagnosis of gross open lesions of the central nervous system was not possible. The culture of cells from the urine of fetuses and neonates had not been reported. A more detailed examination of previous work in each facet of this investigation is given at the beginings of the relevant chapters.

Chapter 2

The Primary Culture of Amniotic Fluid Cells

BACKGROUND

The first documented report of the culture of amniotic fluid cells by Steele and Breg (1970) involved the use of several different types of culture medium and culture vessel. The only conclusions these workers reached were that the best serum supplement was probably fetal calf serum and that the presence of an irradiated feeder layer of fibroblasts increased the number of points of cellular outgrowth. Their method embodied the general principles of subsequent methods. These principles are, the separation of the cells from the amniotic fluid by centrifugation and their transfer to a vessel with tissue culture medium, followed by their incubation at 37°C. The cells then attach to the surface of the vessel and begin proliferation.

Later in 1966 Thiede et al. described a similar method to that of Steele and Breg (1966). In 1967, Jacobson and Barter described the first variation on the basic method of Steele and Breg. They did not centrifuge the amniotic fluid to separate the cells, but added equal volumes of amniotic fluid and tissue culture medium (type unspecified, supplemented with 30% fetal calf serum) to a culture vessel. They allowed the cells to settle for 8 or 24 hours (not clear which) then decanted the medium and unattached cells and debris and added fresh medium. The medium was subsequently changed every three days. Gray et al. (1971) concluded that centrifugation of the amniotic fluid was not necessary, and may even have been detrimental, a view supported by Knörr-Gärtner and Härle

(1972). Gray et al. (1971) used only amniotic fluid as their culture medium, i.e., their method was simply to add amniotic fluid to a culture vessel, incubate and wait for cell proliferation to become "well established". Chitham et al. (1973) failed to culture amniotic fluid cells using amniotic fluid as the culture medium.

Nadler (1968a) described a method which has been slightly modified by a number of workers (e.g., Ferguson-Smith et al. 1971; Nelson and Emery, 1970; Valenti et al. 1969). After centrifugation the cells were resuspended in a small volume of fetal calf serum (in some cases a mixture of amniotic fluid and fetal calf serum), placed in a Petri dish, covered with a coverslip and left for about half an hour before tissue culture medium was added. Petri dish cultures were usually incubated in an atmosphere of 5% CO_2 in air at 37°C.

The maintenance schedule has varied from that of Nadler (1968a) who changed the medium daily, through that of Ferguson-Smith et al. (1971) who changed it every three days, to that of Nelson and Emery (1970) who left the culture undisturbed for an initial period of one week, changed the medium, left it for another week and thereafter changed the medium every three or four days. Other methods have been described but vary only in detail, such as placing the amniotic fluid cells suspended in the fetal calf serum on top of the coverslip rather than beneath it (Abbo and Zellweger, 1970). A more interesting variation was that described by Lee et al. (1970) who claimed that heavy red blood cell contamination

can slow down or completely inhibit cell growth. They lysed the red blood cells by treating the cells from amniotic fluid with ammonium chloride prior to culture.

Attempts have been made to stimulate the growth of amniotic cells in culture by various means, all unsuccessful. Steele and Breg (1966) tried to stimulate growth with phytohaemagglutinin using a method similar to that of Moorhead et al. (1960). Nelson and Emery (1970) tried to stimulate growth with human pituitary extract and steroid hormones. They also tried culture medium taken from a rapidly growing culture and setting up cultures on a collagen substrate. Nadler (1969) reported that the use of human fetal albumin, instead of fetal calf serum, as a medium supplement reduced by 70% the time required for a cytogenetic result. Lisgar et al. (1970) were not able to confirm this. Nelson and Emery (1973) reported a large series of experiments in which they tried to stimulate growth of amniotic fluid cells using different amounts of fetal calf serum, young calf serum, tryptose phosphate broth, chicken embryo extract, thymine, dialysed urine from leukaemic patients and bovine amniotic fluid. None of these variations in culture medium had any effect. Chitham et al. (1973) used human embryo extract to try and improve cell growth but concluded that it had no advantage over chicken embryo extract.

Most workers have favoured a tissue culture medium enriched with non-essential amino acids and vitamins and supplemented with 25 or 30% fetal calf serum. This has

been achieved by using commercially available enriched media such as Ham's F10, by supplementing minimal media such as Eagle's minimal essential medium with the non-essential amino acids and vitamins (e.g., Epstein et al. 1972) or by adding supplements such as bovine embryo extract ultrafiltrate to medium "cocktails" (e.g., Santesson et al. 1969).

The culture vessels used have varied. The majority of workers have, however, favoured Leighton tubes or disposable plastic Petri dishes. Amniotic fluid culture has usually been attempted primarily for cytogenetic studies. As primary growth on a coverslip can be harvested for chromosome preparations, many of the Petri dishes and Leighton tubes have contained coverslips. Nelson and Emery (1973) investigated the use of open and closed culture vessels. They found that "open" Carrel flasks were superior to "closed" vessels but not different from Petri dishes when cell growth at 14 days or less was used as the criterion of success. They concluded that the size of culture dishes and their nature (plastic or glass) did not affect success rates of culture.

The degree of success achieved in the culture of amniotic fluids has varied greatly and has been defined differently by different workers. Perhaps the most liberal definition has been that of Nelson and Emery (1970) who defined success as "the presence of growing cells within three weeks of a culture being set up". In a later paper (Nelson and Emery, 1973) they had reduced their time limit to two weeks. The most

rigorous definition is that of Nadler (1968a) who did not deem a culture successful unless he could "maintain the culture after two subcultures". The first definition is adequate only for the purpose of deciding whether or not amniotic fluid cells have the potential to proliferate in culture but has little relevance to establishing success rates for a diagnostic test such as fetal chromosome analysis. Nadler's (1968a) criterion is too strict if only a cytogenetic result is required as this can be obtained from the primary culture or after the first subculture.

Success rates, by any of these criteria, have varied widely. Steele and Breg (1966) obtained cell growth in 12 of 62 cultures and obtained a chromosome result in two. Nelson and Emery (1970) using their criterion of success obtained cell growth in 49 out of 90 cultures. Jacobson and Barter (1967) had 57 successful cultures out of 85 and achieved chromosome results in 33. Later workers have reported much higher success rates. In a series of 30 pregnancies in which antenatal chromosome studies were undertaken Ferguson-Smith et al. (1971) achieved cytogenetic results in 29, but they required repeat amniocenteses in 5 cases. The same authors were not so successful in culturing a series of 24 samples of amniotic fluid taken at hysterotomy; cell growth was achieved from 21 samples and a karyotype analysis was possible from 17. Therkelsen et al. (1971) karyotyped 41 out of 45 third trimester amniotic fluids and all of 18 second trimester samples. The most remarkable success

rates have been those achieved by Gerbie et al. (1971). They karyotyped 237 out of 250 amniotic fluids taken from 231 patients during 238 pregnancies. Hence they achieved a culture success rate of 94.8% and an overall antenatal diagnosis success rate of 99.6%.

There has been some discussion of the relationship between the stage of pregnancy from which the amniotic fluid is taken and the success rate. Nelson and Emery (1970) reported no difference in success rates between those fluids from pregnancies earlier than 20 weeks gestation and those from later ones. Ferguson-Smith et al. (1971) reported that fluids from pregnancies earlier than 14 weeks and possibly later than 30 weeks were less satisfactory than those obtained from within this range. Nelson (1973[a]) reported that amniotic fluids from severe Rhesus-incompatible cases taken serially throughout the last half of pregnancy required progressively longer to culture and the success rate fell as pregnancy progressed. Nadler and Gerbie (1971) stated that third trimester amniotic fluids collected from women with Rh problems were ".... quite difficult to cultivate". Nelson and Emery (1973) concluded that ".... the main possible factor influencing the changes in growth (of amniotic fluid cell cultures) is serum". They recommended that when "good" batches of fetal calf serum were identified they should be stored and kept for important cultures.

From this survey of published methods, especially the more recent ones, it is clear that there is fairly

general agreement about the following:

1. Petri dishes are the preferred type of culture vessel.

2. 5% CO_2 in air at 37°C is the most widely used incubation condition.

3. Volume and cell count of the amniotic fluid are not closely related to success in culture.

4. The use of vital stains such as trypan blue are of little or no value in assessing the growth potential of amniotic fluid cells.

It is also clear that there is either controversy or lack of adequate data on the following.

1. Does centrifugation have a detrimental effect? Is amniotic fluid itself the best culture medium for growing amniotic fluid cells?

2. What is the best maintenance schedule once a primary culture has been established? Should it be left strictly alone for about one week or should medium changes start 1 to 2 days later?

3. Does the means of or reason for collection or gestational age of the amniotic fluid affect the ability of the cells to grow in culture?

4. What parameters of an amniotic fluid affect the rate at which success is achieved?

Answers to these questions have been sought in the study of amniotic fluid cells in primary culture.

MATERIALS AND METHODS

Amniotic fluids were obtained from a number of sources. These included samples taken from hysterotomy specimens, from women with Rhesus-isoimmunisation, at artificial rupture of membranes via a Drew-Smythe catheter and fluids collected via transabdominal amniocentesis for the purpose of antenatal diagnosis of fetal disease. The methods of collection of amniotic fluid are beyond the scope of this thesis (see Scrimgeour, 1973, for a review of the techniques of collection of amniotic fluid).

The basic method of establishing primary cultures throughout this work was as follows unless otherwise stated.

The amniotic fluid was transported to the laboratory with minimal delay. Relevant details about the fluid, such as gestation of the pregnancy, means of collection, reasons for collection etc. were noted and the fluid was treated as follows:

1. Centrifuged at 1000 rpm for 5 minutes.
2. Supernatant decanted and saved for biochemical studies (Butterworth et al., 1972, 1974).
3. The cell pellet was resuspended in 10 ml. of tissue culture medium and transferred to two 50 mm. plastic Petri dishes which contained four or five 6 x 22 mm. glass coverslips. The cells from about 5 ml. of amniotic fluid were used for each Petri dish. The tissue culture medium was Ham's F10 supplemented with 30% fetal calf serum. Antibiotics incorporated in the

medium were kanamycin (100μg/ml) or penicillin (60μg/ml) and streptomycin (100μg/ml) in combination. (See appendix I for details of plastic and glassware used and preparation of the culture medium.)

4. The cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air (see appendix I for details of method of incubation).

5. Half the medium in each Petri dish was replaced after 2 or 3 days and thereafter half the medium was replaced 3 times per week.

6. The cultures were inspected regularly under the inverted microscope, using either bright field or phase contrast optics, for signs of cell proliferation. When cell proliferation was adequate a coverslip was harvested for chromosome studies (see chapter 4 for details) and/or the culture was subcultured to produce a primary amniotic fluid cell strain (see chapter 3 for details). Cellular morphology was monitored by direct observation with the inverted microscope and by removal of coverslips for fixation in methanol and staining with Giemsa.

The effect of the maintenance schedule was studied by setting up cultures in duplicate as in the standard method. One culture was maintained as in the standard method, the other was not touched for 7 days when half the medium was changed. Half the medium was subsequently changed three times per week. The first culture to produce a cytogenetic result was judged to be the most successful. These methods correspond to

methods III and II respectively in Sutherland et al. (1974b, appendix III). When large volumes of amniotic fluid were available these two methods were tested against the method of Gray et al. (1971) which corresponds to method I of Sutherland et al. (1974b, appendix III).

RESULTS

The results are based on the study of 132 consecutive amniotic fluids received in the two year period 1.12.71 to 30.11.73. Each sample was given a number on arrival (Laboratory number, appendix II). Data on each amniotic fluid received, including reasons for collection, gestational age and some aspects of its behaviour in tissue culture are detailed in appendix II. Some of this data is summarised in table 2.1. Seven of these samples (3 in category vi and 4 in category v) were from anencephalic pregnancies and will not be considered beyond table 2.1 in this chapter. The largest group of amniotic fluids were from Rhesus iso-immunised pregnancies.

Table 2.1. Reasons for collection and gestational ages of the 132 amniotic fluids studied.

Category (appendix II)	Reasons for or means of collection	Number	Gestational Age (weeks) Mean ± S.D.	Range
(i)	Rhesus iso-immunisation	61	27.7 ± 4.8	18-35
(ii)	Hysterotomy specimens	20	16.70 ± 3.5	11-23
(iii)	Antenatal cyto-genetic disease	24	17.0 ± 4.3	14-35
(iv)	Antenatal diagnosis of possible inborn error of metabolism	5	16.2 ± 3.8	14-23
(v)	α-fetoprotein estimation	12	23.3 ± 9.0	12-36
(vi)	Induction of labour	10	36.0 ± 6.0	22-40
Total		132		11-40

Hence, together with samples collected for other reasons the series of amniotic fluids studied spanned the gestational range from 11 weeks to term.

The outcome of the primary cultures is shown in table 2.2 for each category of amniotic fluid. From this table it can be seen that the success rates are high for all categories except iv and vi. In category iv two primary cultures degenerated, one leading to failure of an antenatal diagnosis (42) and the other (284), which was grossly blood-stained, required a repeat amniocentesis one week later which resulted in a successful culture. Except for two cultures lost to primary contamination and another to secondary contamination before assessment, category vi contained the only primary cultures in the whole series which did not show any sign of cellular proliferation.

The quality of success can be measured by the time taken to achieve a cytogenetic result (when this was done in the minimum time) and by the time in primary culture. Unfortunately, both these times are subjective in that they rely on the experience of the operator and are not continuous because cytogenetic harvesting was usually only done twice a week and sub-culturing three times per week. However, since almost all these operations were done by one person the only trend is perhaps a shortening of the time taken to achieve a cytogenetic result as a proportion of the time in primary culture. As there is no other measurement of the quality of success achieved, the times to a

Table 2.2. Numbers of primary amniotic fluid cultures in each category which were harvested for cytogenetic studies in the minimum time required and the number which were subcultured, with reasons why some were not subcultured.

Category (appendix II)	Number	No. harvested for cytogenetic studies in minimum time	No. subcultured	Reasons for failure to subculture (Laboratory number, appendix II, table I)
(i)	61	28	58	Bacterial contamination (No. 214). Culture degenerated after cytogenetic studies (Nos. 180 and 207).
(ii)	20	16	18	Culture degenerated after cytogenetic studies (No. 129). Culture degenerated before cytogenetic studies (No. 43).
(iii)	24	23	18	Bacterial contamination after cytogenetic result (Nos. 216, 220, 226, 227, 249). Culture degenerated before cytogenetic studies (No. 227).
(iv)	5	0	3	Culture degenerated (Nos. 42, 284).
(v)	8	7	8	
(vi)	7	1	3	Cultures showed no signs of cellular proliferation (Nos. 108, 169, 267). Amniotic fluid contaminated at collection (No.299).
Total	125	75	108	

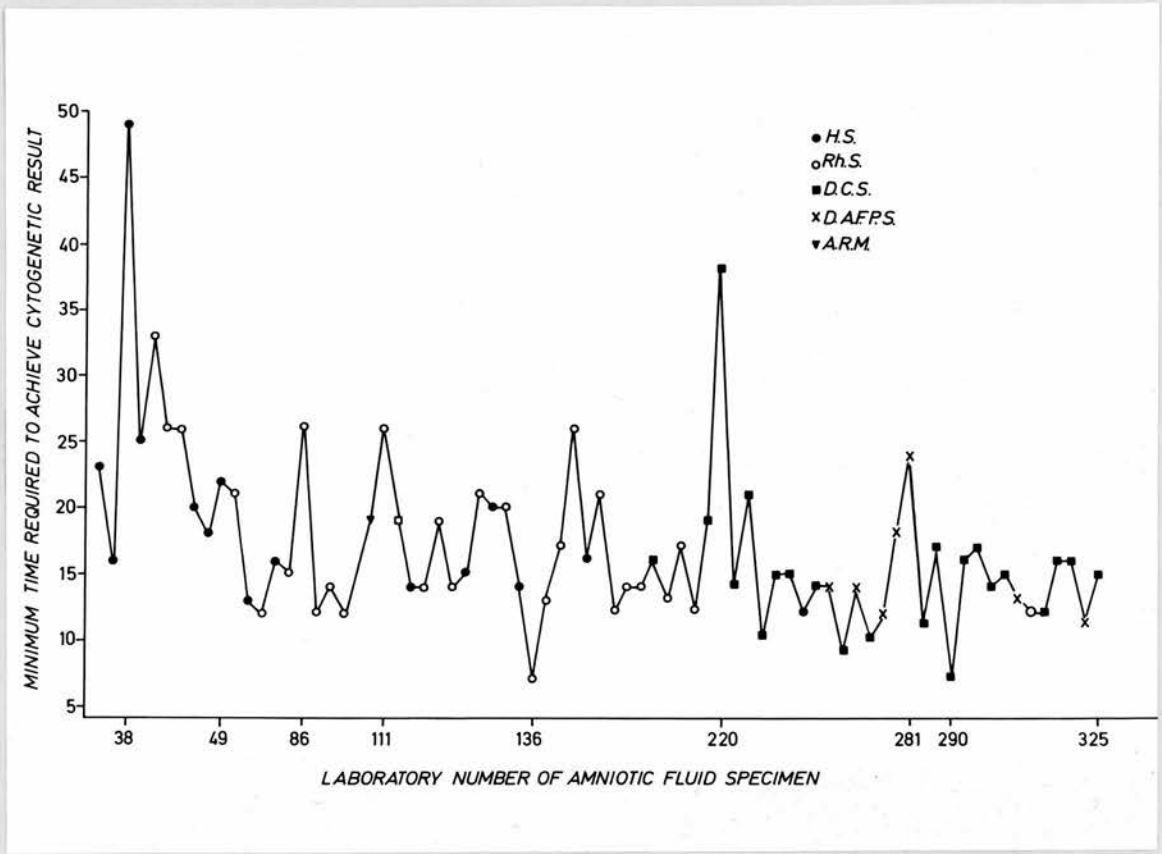


Fig. 2.1. Minimum times required to achieve a cytogenetic result from amniotic fluid samples in the sequence in which they were received. (H.S. - hysterotomy specimen; Rh.S. - Rhesus iso-immunised specimen; D.C.S. - diagnostic cytogenetic specimen; D.A.F.P.S. - diagnostic α-fetoprotein specimen; A.R.M. - specimen collected at induction of labour at term).

cytogenetic result and the times in primary culture of amniotic fluid cultures in each category are shown in table 2.3 and 2.5.

The time to chromosome result data shall be considered first. From Fig. 2.1 it can be seen that after the first 10 or so samples there was a fall in the time required for such a result. This most probably represents early inexperience in obtaining chromosome preparations from amniotic fluid cultures. After this initial fall there were marked fluctuations in the time required from 7 days (136) up to 38 days (220). These fluctuations appeared

Table 2.3. Times taken to achieve a cytogenetic result from 75 amniotic fluid cultures according to category of sample.

Category	No.	Days to cytogenetic result		
		Mean	± S.D.	Range
i	28	17.4	± 6.2	7-33
ii	16	19.2	± 8.8	12-49
iii	23	15.1	± 6.0	7-38
v	7	15.1	± 4.5	11-24
vi	1	19		-
Total	75	16.9	± 6.7	7-49
Total - first 10	65	15.5	± 5.0	7-38
First 10	10	26.0	± 9.4	16-49

to be random around a mean of about 15 days with a few marked deviations down and slightly more up.

The times taken to achieve cytogenetic results have been analysed according to the category of the amniotic fluid (Table 2.3). With the exception of category vi which only contained one result, Student's t-tests were carried out between each group and the values obtained are shown in table 2.4. The figures from this table and table 2.3 indicate that apart from the first 10 results which took longer to obtain than those for any of the other groups, the origin of the amniotic fluid does not affect the time taken to achieve a cytogenetic result. The significant difference between the results from the hysterotomy specimens and the last 65 samples is artefact. Seven of the hysterotomy samples were included in the first 10 results; if these 7 are excluded from the

Table 2.4. Student's t-test scores from comparisons of the times taken for a cytogenetic result according to the category of the sample.

Category	(i)	(ii)	(iii)	(v)	Total	Total - first 10
(ii)	0.776	-				
(iii)	1.332	1.714	-			
(v)	.911	1.145	N.D.	-		
Total	0.359	1.169	1.139	0.682	-	
Total - first 10	1.554	2.217*	N.D.	N.D.	1.355	-
First 10	N.D.	N.D.	N.D.	N.D.	3.752**	5.271**

* P <.05
** P <.001 N.D. - not done

hysterotomy series and the t value recalculated then it is no longer significant (t = 0.384). Since this first group of 10 results are different from the remaining 65 they will be excluded from further consideration. Although chromosome results were not attempted in the minimum time from all cases, there was no conscious selection involved. All cases where the result was of clinical significance were completed in the minimum time, whether this was done for other cases depended on pressure of work, vacations etc., not the behaviour of the culture.

There were no significant correlations between gestational age and time to result either within the categories or for the group as a whole. In addition, the time to result from fluids collected from pregnancies of 20 weeks' gestation or less were not significantly different from that required from pregnancies of more

Table 2.5. Times in primary culture for 107 amniotic fluid cultures according to category of sample.

Category	Number	Days in primary culture		
		Mean	± S.D.	Range
(i)	58	28.57	± 8.1	18-55
(ii)	18	27.61	± 7.8	18-49
(iii)	18	25.83	± 4.3	17-34
(iv)	3	25.67	± 4.5	21-30
(v)	8	24.3	± 6.3	19-36
(vi)	3	29.0	± 2.6	27-32
Total	108	27.56	± 7.2	17-55

than 20 weeks' gestation.

The times in primary culture according to category are shown in table 2.5. The times in primary culture did not show the same initial decline as did the times to chromosome result. Hence it was not necessary to exclude any cultures from consideration. Student's t-tests were carried out between some of the categories and the values are shown in table 2.6. None of these values are significant. Hence time in primary culture was unrelated to the origin of the amniotic fluid. In addition there was no correlation between gestational age of the amniotic fluid and time in primary culture.

The data were examined to ascertain whether there was any relationship between the cellular content of the amniotic fluid and the time to chromosome result or the time in primary culture. The cell counts of the amniotic fluids ranged from less than 1000 cells/ml

Table 2.6. Student's t-test scores from comparisons of the times taken for a cytogenetic result according to category.

Category	(i)	(ii)	(iii)	(v)
(ii)	0.443	-		
(iii)	1.373	0.846	-	
(v)	1.450	1.072	0.748	-
Total	0.818	-	0.984	1.259

(these were arbitrarily given a value of 1000 cells/ml. as this was the lower limit of sensitivity of the method used - see appendix I) up to 200,000 cells/ml. In spite of this range the cell count was not related to the behaviour of the primary culture.

The availability of serial samples of amniotic fluid collected from Rhesus iso-immunised patients afforded the opportunity of studying the effect of this disease on the growth characteristics of amniotic fluid cells. The behaviour in primary culture of amniotic fluid cells derived from eight such patients are shown in table 2.7. There is no significant correlation between the O.D.D. and the time to a chromosome result or to the time in primary culture. By inspection the time to chromosome result is obviously unrelated to both O.D.D. and gestation. The data are not clear for the time in primary culture. The results for patients 1 and 5 show a progressive increase in time in primary culture associated with a progressive decrease in O.D.D. Patient 1 had an affected child whereas patient 5 had an

Table 2.7. Behaviour in primary culture of serial samples of amniotic fluid collected from eight patients.

Patient Number	Gestation (weeks)	Days to chromosome result	Days in 1° culture	O.D.D.*
1	18	-	28	0.16
	22	26	24	0.20
	24	12	20	0.18
	33	21	49	0.08
	35	19	36	0.08
2	23	-	18	.16
	26	-	26	.28
	28	14	28	.50
3	19	7	31	0.12
	30	13	20	0.07
	33	26	-	0.05
4	24	-	34	0.20
	30	-	27	0.11
	32	14	43	0.11
5	20	17	20	0.14
	23	21	28	0.14
	26	13	31	0.08
	29	-	36	0.06
	33	30	41	0.04
6	20	12	43	0.12
	27	12	30	0.07
	30	-	33	0.05
7	19	-	18	0.09
	30	-	26	0.11
	32	-	26	0.11
8	27	12	29	0.12
	34	26	27	0.27

* Optical density difference at 450 mμ (Robertson, 1969).

unaffected child. Patient 2 had a severely affected child, but in this case the time in primary culture increased with a corresponding increase in O.D.D. More such series of amniotic fluids would have to be studied to determine which, if any of these apparent associations

are significant.

There was little opportunity to study the effect of gross blood contamination of the amniotic fluid on the growth of the primary cultures. Only 6 heavily blood-stained samples were received and two of these were from the same patient. The first (284) showed poor growth in culture but the second (286), collected a week later, and just as heavily blood-stained was cultured successfully. The main problem associated with blood-stained samples in primary culture was that the red cells made observation of the culture with the inverted microscope difficult. These red cells were progressively removed by medium changes and after about two weeks no longer interfered.

The results of the comparison of the two schedules of maintenance during the first week of primary culture (methods II and III, Sutherland et al., 1974b; appendix III) are shown in table 2.8. The differences between the two methods are not significant either for the time taken to achieve a cytogenetic result or the time in primary culture. It is, however, noteworthy that the duplicate cultures showed considerable variation. For 269, one culture took more than twice as long as the other to achieve a cytogenetic result and for 123 one culture degenerated even though both achieved cytogenetic results in the same time. Early in this work it became apparent that the initial maintenance schedule was of little importance to the success of the culture and, apart from the experimental cultures, the first medium change was usually carried out after three to five days,

Table 2.8. Times taken to achieve a cytogenetic result and times in primary culture for duplicate cultures using maintenance methods II and III.

Number	Days to cytogenetic result		Days in primary culture	
	Method III	Method II	Method III	Method II
89	18	12	33	29
94	15	15	25	28
115	19	19	36	29
119	14	16	20	20
121	14	18	20	23
123	21	20	49	*
127	22	19	38	36
176	17	19	20	24
200	16	18	30	30
246	14	10	28	26
247	15	15	26	28
260	14	20	18	27
261	10	10	18	26
263	15	10	25	21
268	13	13	26	28
269	10	24	30	39
271	15	15	25	34
274	9	9	17	17
276	10	11	32	20
277	9	10	32	20
Mean ±S.D.	14.5 ±3.7	15.2 ±4.4	27.0 ±8.0	26.8 ±5.6

* Culture degenerated and could not be subcultured.

whenever most convenient.

The results of the study on a large volume of amniotic fluid from which 15 primary cultures were set up using the three methods outlined in Sutherland et al. (1974b, appendix III) are shown in table 2.9. Cytogenetic preparations were not actually harvested from all these cultures and the time to cytogenetic result was the time at which there were sufficient cells on a coverslip which could have been harvested. It can be seen from these results that there is little difference between methods II and III but that method I is inferior to these two. These results are valid for only this one sample

Table 2.9. Time taken to achieve a cytogenetic result and time in primary culture for 15 cell strains set up from one sample of amniotic fluid using three different methods.

Method and cell strain designation		Days to cytogenetic result	Days in primary culture
	a	22	43
	b	*	*
I	c	23	38
	d	21	36
	e	20	36
	f	14	25
	g	15	28
II	h	14	25
	i	12	22
	j	16	22
	k	14	22
	l	11	23
III	m	11	22
	n	11	22
	o	11	22

* Culture b showed only minimal evidence of growth, degenerated before there were sufficient cells for a chromosome result and could not be subcultured.

of amniotic fluid. It was not possible to obtain other large volumes of amniotic fluid to repeat this experiment.

Studies on the morphology of the cells seen in primary cultures of amniotic fluid have been published (Sutherland et al., 1974a, appendix III).

DISCUSSION

The main feature of these results is the high success rate attained for the culture of amniotic fluid cells. Much early work on amniotic fluid cell culture implied that these cells were difficult to culture and success rates were low. Nelson and Emery (1971) successfully cultured only 49 out of 90 amniotic fluids. Success rates and times taken to achieve chromosome results in the early series have been reviewed by Nelson (1973a). In table 2.10 the overall success in the present series is compared with series published in 1971 or later which included more than 20 amniotic fluid cultures. Success rates ranged from 55 to 100% but those series with the highest success rates were both small. The low overall success rates in some series (Robinson et al., 1973; Turnbull et al., 1973) were mainly due to failures in the early part of the series; both these series had success rates greater than 90% towards the end. The success rate of the present series compares very favourably with other series, especially since four of the failures were from 7 amniotic fluids collected at the induction of labour. Other series did not have such samples included in them with the exception of Mulcahy and Jenkyn (1973) whose series included 4 samples collected at term.

In view of the high success rate any attempt to study the effect of various treatments on the growth of amniotic fluid cells had to be assessed in terms of time to a cytogenetic result or time to subculture. Most series do not contain data on these parameters of cell growth.

Table 2.10. Series of amniotic fluid cultures which have been published in 1971 or later and which included more than twenty cultures.

Authors	No. in series	No. successful	% successful	Comments
Ferguson-Smith (1971)	35	29[a]	83	diagnostic series
	24	21[a]	88	hysterotomy series
Gerbie et al. (1971)	250	238[a]	95	
Gray et al. (1971)	40	40[a]	100	
Epstein et al. (1972)	24	21[a]	88	
Therkelsen et al. (1972)	18	18[a]	100	second trimester
	45	41	91	third trimester
Wallace (1972)	22	18	82	2 failures due to contamination
Cederqvist et al. (1973)	50	49[a]	98	
Hsu et al. (1973)	c.200	? [a]	c.91	actual figures not given
Milunsky (1973)	220	210[a]	96	
Mulcahy and Jenkyn, (1973)	18	18	100	diagnostic
	23	19	83	experimental
Prescott et al. (1973)	44	40[a]	91	
Robinson et al. (1973)	171	119	70	54 of last 55 successful
Turnbull et al.	141	79	56	experimental to March 1972
	73	40	55	diagnostic to March 1972
	29	27	93	diagnostic April 1972 to 1973
Present Series	125	120	96	(from table 2.2)

[a] - success defined as cytogenetic result, otherwise success defined as the observation of cell growth.

Only five of the series in table 2.10 have details of the time required to achieve a cytogenetic result and these are shown in table 2.11. The shortest time required was by Gray et al. (1971). The only other report of such rapid results being consistently obtained was by Lisgar et al. (1970) who required an average of 8.3 days (range 3-26) to achieve cytogenetic results from 19 amniotic fluid cultures. Cederqvist et al. (1973) did not obtain their results in the minimum time but routinely harvested for cytogenetic studies after 14 days. Epstein et al. (1972) required from $2\frac{1}{2}$ to $5\frac{1}{2}$ weeks (mean 4 weeks) early in their series but this time fell to about three weeks towards the end of the series. Therkelsen et al. (1972) found that the time required decreased as their series progressed. This decrease in time, also found in the

Table 2.11. Minimum times required to produce cytogenetic results from amniotic fluid cell cultures.

Series	No. of fluids	Days to result		Comments
		Mean	Range	
Gray et al. (1971)	40	7	5-10	
Ferguson-Smith et al. (1971)	29	18.4	7-31	
Therkelsen et al. (1972)	17	10.5	?	second trimester
	14	12.8	?	third trimester
Mulcahy and Jenkyn (1973)	18	22.8	17-34	diagnostic
	19	22.0	15-38	non-diagnostic
Prescott et al. (1973)	40	25.9	14-44	
Present series	65	15.5	7-38	(table 2.3, page 35)

present series, probably represents an increase in the expertise of the operator rather than any increase in the growth rate of the cells especially where the techniques used are not changed.

There are no data in the literature on the time in primary culture for amniotic fluid cells. This may well vary with the growth surface area of the culture vessel used. Presumably cultures initiated in, say, small Leighton tubes would be ready for subculture before those grown in Petri dishes ot T-flasks. Possibly because of this lack of data on time in primary culture there is little information on degeneration of primary cultures. This was the main reason for failure of primary cultures in the present series, apart from microbial contamination. Most early reports of amniotic fluid cell culture indicated that more cultures showed cellular proliferation than yielded cytogenetic results. Nadler (1968[a]) reported that 3 out of 27 primary cultures, which grew well, could not be successfully subcultured. Turnbull et al. (1973) reported that although 40% of the first 241 fluids they studied showed growth in culture only 25% grew well enough to produce a cytogenetic result; this presumably means that the cultures degenerated. The reasons why cells cease to divide once they have started remain unknown but inadequate culture conditions is one possibility.

The behaviour of the amniotic fluid cells in primary culture was independent of the cell count, gestation, and the reason for which the fluid had been collected. The

one exception was the difficulty experienced in culturing cells from term amniotic fluids. Mulcahy and Jenkyn (1973) were only able to culture two out of five samples of amniotic fluid collected at term. This difficulty with such fluids may be due to contamination with obstetric antiseptics (Scrimgeour, personal communication) or to the build up of waste products in amniotic fluid towards term, producing a situation similar to that for urine (see discussion chapter 8). Samples grossly contaminated with blood were found to grow as well as other samples, a finding in agreement with Gerbie et al. (1971) and Robinson et al. (1973) but not with most other authors. The degree of severity of Rhesus iso-immunisation (as expressed by the O.D.D. at 450 mμ) had no consistent effect on the quality of success of cell culture. This finding does not agree with most other published series in which such fluids were included, but these were mainly early series with lower success rates. Nelson (1973[a]) reported that repeated samples from severely Rhesus iso-immunised pregnancies take progressively longer to grow in culture. Nadler and Gerbie (1971) found cells from third trimester Rhesus iso-immunised pregnancies difficult to cultivate. Further series of such fluids would need to be studied to refute these claims but the present series lends them no support.

Studies on the morphology of the cells in amniotic fluid cultures are presented and discussed in Sutherland et al. (1974a, appendix III). The only additional study of this nature is that of Kaback and Leonard (1972)

whose findings are essentially similar to those in the present series.

The experiments on the maintenance schedule of the cultures indicated that this did not affect the rate of cell growth. The wide variations in the maintenance schedule amongst published methods of primary amniotic fluid cell cultures suggest that this is certainly not an important consideration.

The effect of lack of centrifugation of the amniotic fluid and the consequent use of amniotic fluid alone or as a high proportion of the culture medium was not adequately investigated due to the lack of suitable material (large volumes of amniotic fluid). The only fluid which was studied in this regard did not support the use of amniotic fluid alone as a culture medium (Gray et al. 1971). Other methods have since been published which do not include centrifugation (Cederqvist et al., 1973; Knörr-Gärtner and Härle, 1972) but these do not use amniotic fluid alone as a culture medium. Nakagome et al. (1972) did not centrifuge but found that using amniotic fluid alone as the culture medium for longer than "overnight" before replacing it with more conventional culture medium was "less satisfactory". Hence only Gray et al. (1971) have obtained good results using amniotic fluid itself as a culture medium. Furthermore, no others have produced data which indicate that centrifugation of amniotic fluid, as carried out in many primary culture methods, is detrimental to cell growth. The only others who have achieved cytogenetic

results as quickly as Gray et al. (1971) are Lisgar et al. (1970) who centrifuged their amniotic fluids at 250g for 15 minutes. Further controlled studies similar to the one performed on fluid 264 are required to establish whether centrifugation is harmful and whether amniotic fluid alone is an adequate culture medium. Since the experiment on 264 did not test lack of centrifugation in combination with the use of conventional tissue culture medium, this experiment should be redesigned such that the three groups of cultures would be (a) no centrifugation and amniotic fluid alone as a culture medium for one week, (b) no centrifugation but equal parts amniotic fluid and culture medium and, (c) standard method.

The results of the studies reported in this chapter, together with those in the literature indicate that cellular proliferation should be observed in virtually 100% of amniotic fluid cell cultures provided that the fluid is not collected at induction of labour. For reasons which remain unknown about 5% of cultures will degenerate in the primary culture, some leading to failure of cytogenetic studies and all failing to produce cell strains. All attempts to stimulate amniotic fluid cells to more rapid growth have failed. The times taken to achieve cytogenetic results will decrease as the operator becomes more experienced in handling these cultures. The rate at which cultures proliferate is probably dependent on close attention to the various details of tissue culture technology rather than on any single factor.

SUMMARY

The behaviour of 125 amniotic fluid samples in primary culture has been studied. The times required to achieve cytogenetic results in the minimum time was established in 75 cases. The primary cultures were subcultured to produce 108 amniotic fluid cell strains. The behaviour of the amniotic fluids in tissue culture has been analysed with respect to reason for collection of the fluid, gestational age, cell count and degree of Rhesus iso-immunisation. Variations in the method of establishing the primary cultures were examined. The morphology of the cells which proliferate in these primary cultures of amniotic fluid have been studied.

Chapter 3

The Serial Cultivation of Amniotic Fluid Cell Strains

BACKGROUND

The classic papers of Hayflick and Moorhead (1961) and Hayflick (1965) finally dispelled long-held ideas that human diploid cell strains were immortal (Medawar, 1958). This concept of immortality was supported by reports (e.g., Paul, 1965) that Carrel had maintained a cell strain, derived from chick embryo heart, in serial culture for 34 years. Hayflick (1965) has suggested that this "cell strain" only survived because it was being supplemented with new cells in the chick embryo extract component of the culture medium. (Paul, 1965, gives a detailed account of the early development of tissue culture.)

It is now accepted that human diploid cell strains have a limited life *in vitro*, compared with cell lines which have unlimited lives *in vitro*. Criteria for differentiating between cell lines and strains have been presented by Hayflick and Moorhead (1961). It should be noted that the concept of the "Hayflick limit" has been challenged. Moore and McLimans (1968) claim that lymphoblastic cell strains qualify as diploid, are not transformed, and can be cultured indefinitely. Hay (1970) has suggested that the apparent limited life may be due to inadequate tissue culture conditions. The ability of Litwin (1972) to increase the number of passages to which cell strains can be cultivated by supplementing the culture medium with excess tyrosine supports Hay's suggestion.

There is no report in the literature which documents

the behaviour of amniotic fluid cell strains in prolonged serial cultivation. Nadler and Gerbie (1970) implied that they could not subculture 25% of 155 successful primary amniotic fluid cell cultures more than three times. Melancon et al. (1971) stated that amniotic fluid cell cultures contained two cell types, an epithelial type which can be subcultured only two to five times and a fibroblast-like cell which can be subcultured for more than 30 passages. Littlefield (1971) has claimed that " .... six to eight weeks of rapid culture can approach the limit of the growth potential of amniotic fluid cells". Gray et al. (1971) established cell strains from 23 amniotic fluids which had been subcultured from one to twelve times and " .... continue to grow vigorously". Wallace (1972) found that of the cell types present in primary amniotic fluid cultures only fibroblast-like cells " .... grew luxuriantly and could be successfully passaged".

The lack of information on the behaviour of amniotic fluid cell strains prompted this study. A knowledge of the behaviour of these cell strains is important as biochemical assays may require large numbers of cultured cells. In the antenatal diagnosis of an inborn error of metabolism this may mean that the cell strain has to be subcultured three or four times before there are sufficient cells for a biochemical assay. It is essential to know the time required for, and the probability of, producing enough cells for a biochemical diagnosis. As Brock (1973) when discussing antenatal diagnosis commented

" .... success to the biochemist is more elusive than success to the cytogeneticist".

MATERIALS AND METHODS

The cell strains were developed by subculturing the primary cultures described in chapter 2.

There were three main aspects of this study of the serial cultivation of amniotic fluid cell strains:

1. Observations of the behaviour of these cell strains when serially cultured until senescent. This included studies of cell morphology and karyology. The methods are as in Sutherland et al. (1974a, appendix III). It should be noted that those cell strains described in Sutherland et al. (1974a, appendix III) were serially cultured using Ham's F10 tissue culture medium with 30% fetal calf serum supplement. The cell strains cultured since that series was completed had the fetal calf serum supplement in the culture medium reduced from 30% to 15%.

2. Measurement of the time required to produce enough cells for a biochemical assay was carried out. The methods are as in Sutherland and Bain (1973), appendix III. The cell strains in this published series were grown with a 30% serum supplement in the culture medium except for the last 15 in the series where a 15% serum supplement was used. All subsequent cell strains were grown in medium with a 15% serum supplement.

3. Two experiments were set up to study the effect of the amount of serum supplement and type of culture vessel on the behaviour of the cell strains. Duplicate cultures of two amniotic fluids (numbers 246 and 247, appendix II) were set up. The cultures were split as in Fig. 3.1 to produce six substrains from each amniotic

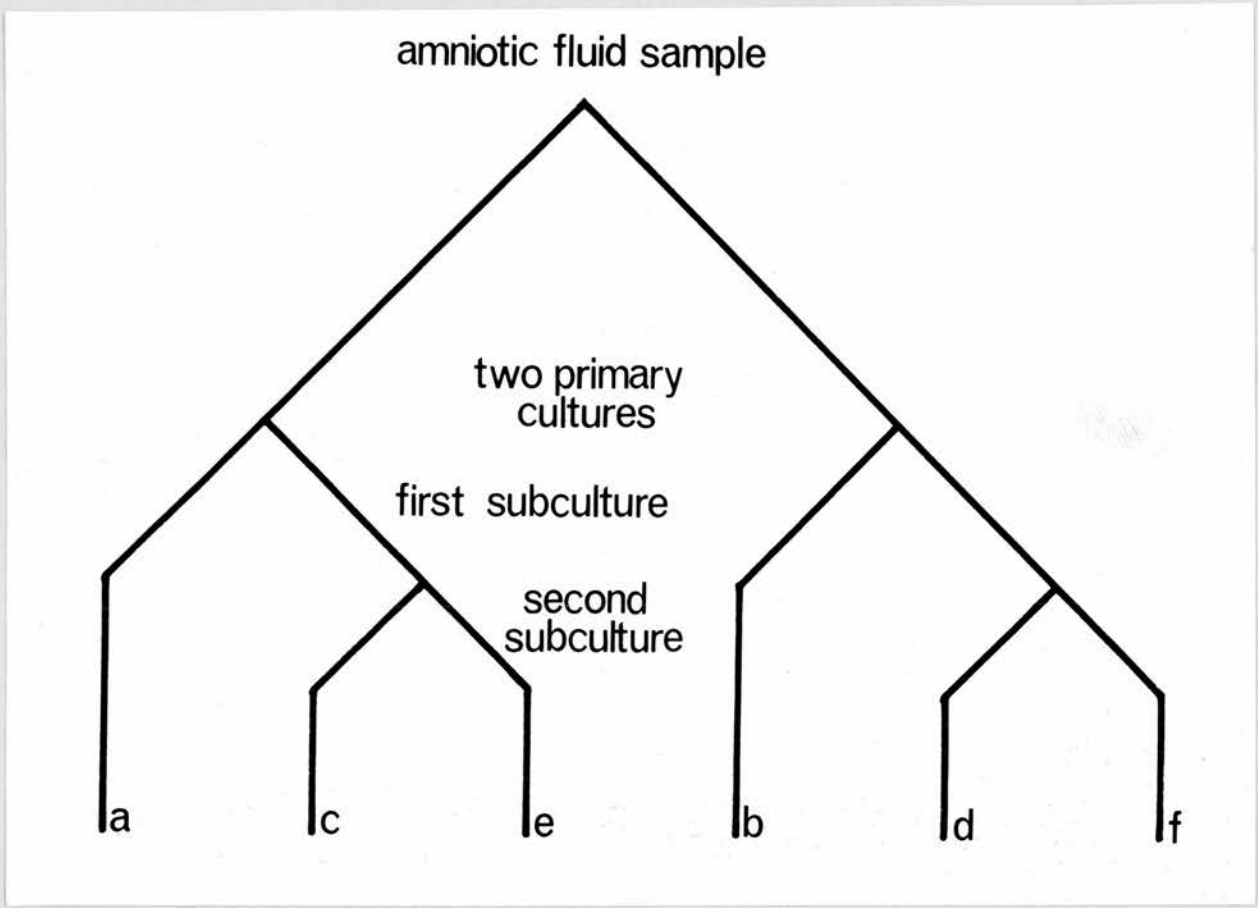


Figure 3.1. Schematic representation of the way six cell strains were obtained from amniotic fluids number 246 and 247. Substrains a and b were cultured in baby feeding bottles using medium with a 15% serum supplement. Substrains c and d were similarly maintained but a 30% serum supplement was used. Substrains e and f were maintained in T-flasks using medium with a 30% serum supplement.

fluid. Four were serially cultured in baby feeding bottles, two using medium with a 30% serum supplement and two with a 15% serum supplement. Two were serially cultured in Nunclon T-flasks with a growth surface area of 25 cm^2. When the cells in the first T-flask were subcultured the flask was not discarded but fresh culture medium was added to it and it was incubated so that the cells not removed could continue growth. When the cells in this flask became confluent the procedure was repeated

and the cells removed with trypsin were used to prepare a flying coverslip. This routine was performed until the cells would no longer grow to confluency. Hence the one culture flask was maintained over several months with those cells which could be readily removed with trypsin being continually discarded. This produced a cell strain with cell types which were "trypsin resistant".

One large volume of amniotic fluid (264) was used to set up 15 primary cultures using three different methods (Sutherland et al., 1974b, appendix III). This allowed comparison of the three methods (see chapter 2) and was intended for use to study the reproducibility of the various components of cell behaviour reported in this chapter.

RESULTS

The results of the first series of cultures on which long term studies were completed are presented in Sutherland et al. (1974a, appendix III). This report covered samples numbered from 47 to 127 inclusive (appendix II), and will henceforth be referred to as the first series. Samples prior to number 47 were excluded from the first series because of inexperience in tissue culture. Those samples not included in Sutherland et al. (1974a), but coming into the consecutive series from 47 to 127, and the reasons for their exclusion are shown in table 3.1. Hence the first series of 31 cell strains were derived from 41 consecutive amniotic fluids.

All tissue culture was discontinued for a short period after the detection of mycoplasma contamination. After resumption of tissue culture, mycoplasma testing was

Table 3.1. Reasons for exclusion of 10 amniotic fluid cell strains from the first series.

Reason	Laboratory Numbers (appendix II)	Total
Cell strain not cultured to senescence	49, 110	2
Cell strain had possible inborn error of metabolism	62	1
Cell strain lost to bacterial contamination	64, 76	2
In vitro chromosome changes	70, 115	2
Mycoplasma contamination	111, 122	2
Primary culture showed no signs of cell growth	108	1

consistently negative until cell strain number 264 had been in culture for some time. Hence the cultures in the second series, on which long term studies had been completed, were numbered from 152 to 247. There were 35 amniotic fluids in this series, but there were only 18 cell strains on which long term studies were completed. The reasons for exclusion of 17 are shown in table 3.2. As can be seen from this table there was a problem with microbial contamination. This was due to a Gram negative organism in a batch of medium. Routine sterility testing failed to detect this organism before the medium was used. All cultures in this particular series lost to microbial contamination were in this one episode.

Since there were only 4 cell strains in this second series of 18 which did not originate from Rhesus iso-

Table 3.2. Reasons for exclusion of 17 amniotic fluid cell strains from the second series.

Reason	Laboratory Numbers (appendix II)	Total
Cell strain not cultured to senescence	181, 186, 231	3
Cell strain had an inborn error of metabolism	205	1
Cell strain lost to microbial contamination	209, 210, 213, 224	4
Primary culture lost to microbial contamination	207, 214, 216, 220, 226, 227	6
Primary culture could not be subcultured	217, 180	2
Primary culture showed no signs of cell growth	169	1

immunised fluids, all 18 will be considered together. The justification for this is that in the first series the origin of the amniotic fluid did not affect the behaviour of the cell strains in culture. The properties of the cell strains in the second series have been analysed similarly to the first series. The 18 cell strains were derived from amniotic fluid samples collected from 13 women, i.e., one woman contributed 3 samples and three women contributed 2 samples each. Seventeen of the cell strains had normal karyotypes, chromosome studies were not carried out on the cells grown from one sample (fluid number 168). Table 3.3 compares some features of the amniotic fluids in the two series. As can be seen there is no significant difference between the mean gestational ages in the two series. The sex ratios are reversed from the first series to the second but this is obviously due to the small numbers involved as the combined sex ratio is not

Table 3.3. Gestational ages and chromosomal sexes of the amniotic fluids which yielded cell strains in the first and second series.

Series	Number in Series	Gestation (weeks)	Chromosomal Sex	
			Male	Female
First	31	25.6[a] 11-39[b]	12	19
Second	18	25.7[a] 15-40[b]	11*	6*

a - mean; b - range.

* - chromosome studies not carried out on one sample.

Table 3.4. Data on the amniotic fluid cell strains cultured to senescence in the two series.

Series	No. of passages	Days in 1° culture	Days as a cell strain	Mean subculture interval (days)
1	13.90 ± 6.52[a]	26.32 ± 5.43	91.94 ± 35.01	7.19 ± 2.07
	3-29[b]	18-41	19-146	3.0-12.0
2	15.67 ± 15.76	32.00 ± 10.15	101.67± 54.35	8.98 ± 3.22
	2-55	20-55	25-248	3.8-14.4

[a] - mean ± standard deviation.
[b] - range.

different from unity.

Table 3.4 compares some properties of the cell strains in the two series. The differences between the two series are not significant for the number of days as a cell strain (interval between subculture of the primary culture and death of the cell strain) or for the number of passages for which the cell strain could be cultured prior to death. The number of days in primary culture was significantly longer ($P < .05$) for the second series. The mean interval between subcultures was also significantly longer ($P < .05$) for the second series than for the first series.

Similarly to the first series, there was no correlation between gestational age and time in primary culture, number of passages or the mean subculture interval. There was however a significant ($P < .05$) correlation ($r = -0.48$) between the time in primary culture and the number of passages. The significance of this correlation is due to the results for laboratory numbers 190 and 247 which, statistically, can be regarded

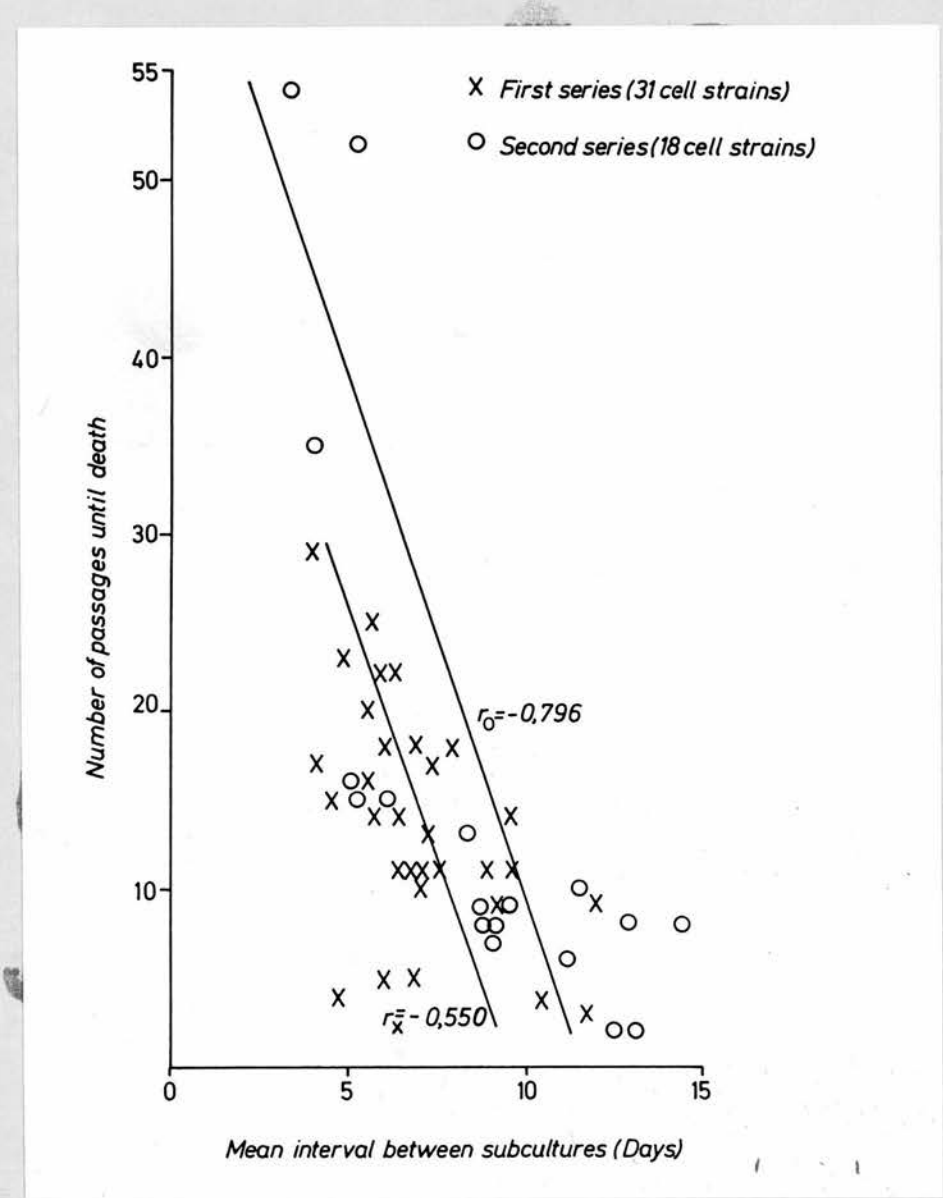


Fig. 3.2. Relationship between the number of passages for which a cell strain could be cultured prior to death and the mean interval between subcultures.

as outliers. If the results for these two cell strains are omitted from the calculation the correlation is no longer significant, being in agreement with the first series. As in the first series the correlation between number of passages and mean interval between subculture is significant ($r = -0.796$; $P < .001$). This relationship is shown for both series in Fig. 3.2. Since the growth

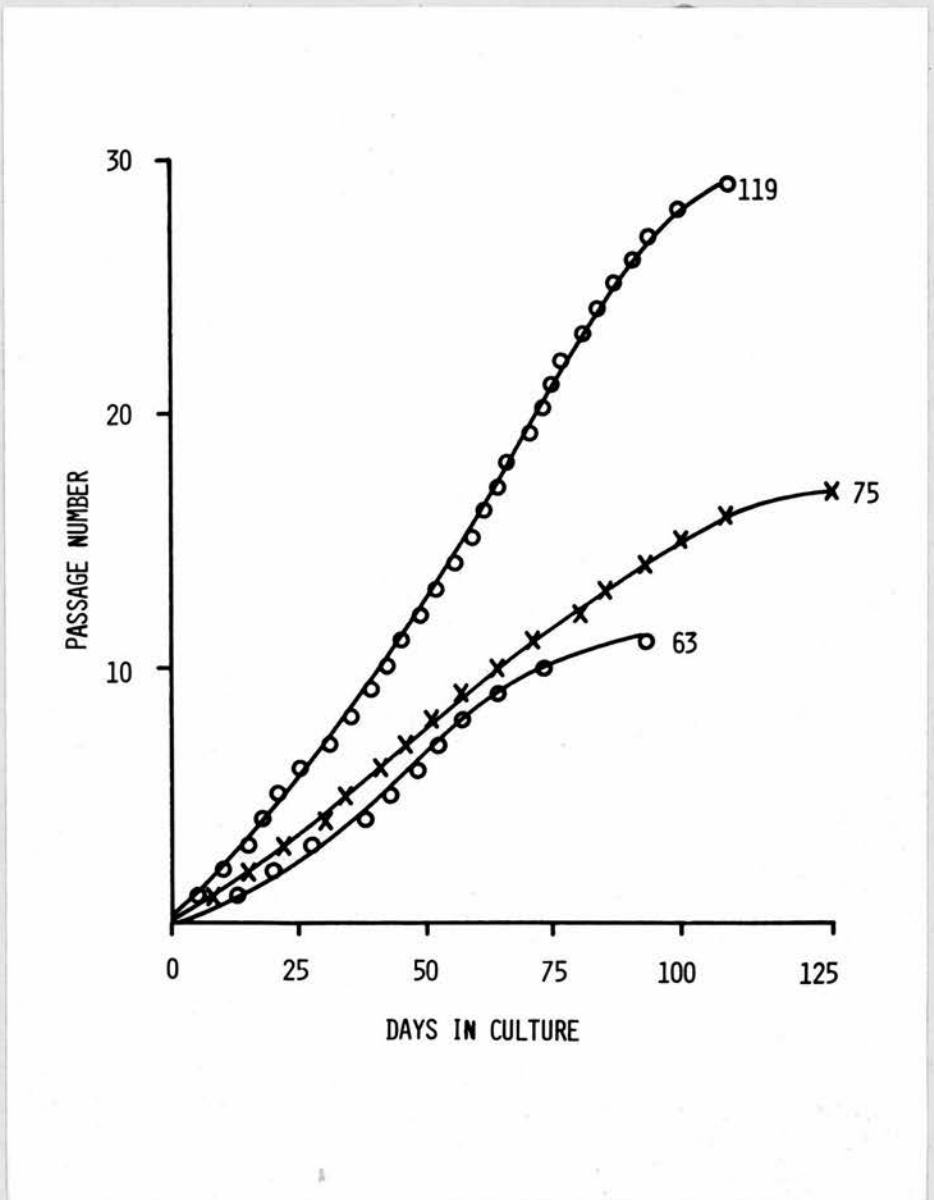


Fig. 3.3. Growth curves of three amniotic fluid cell strains which are typical for longer surviving (119) and intermediate surviving (75 and 63) cell strains.

curve for amniotic fluid cell strains is not linear, showing an initial lag phase and a final slowing down (Fig. 3.3) it is possible that the calculation of mean interval between subcultures as carried out produces this correlation artefactually. In addition those cell strains which survive only for a small number of passages may unduly influence this correlation. Hence the mean

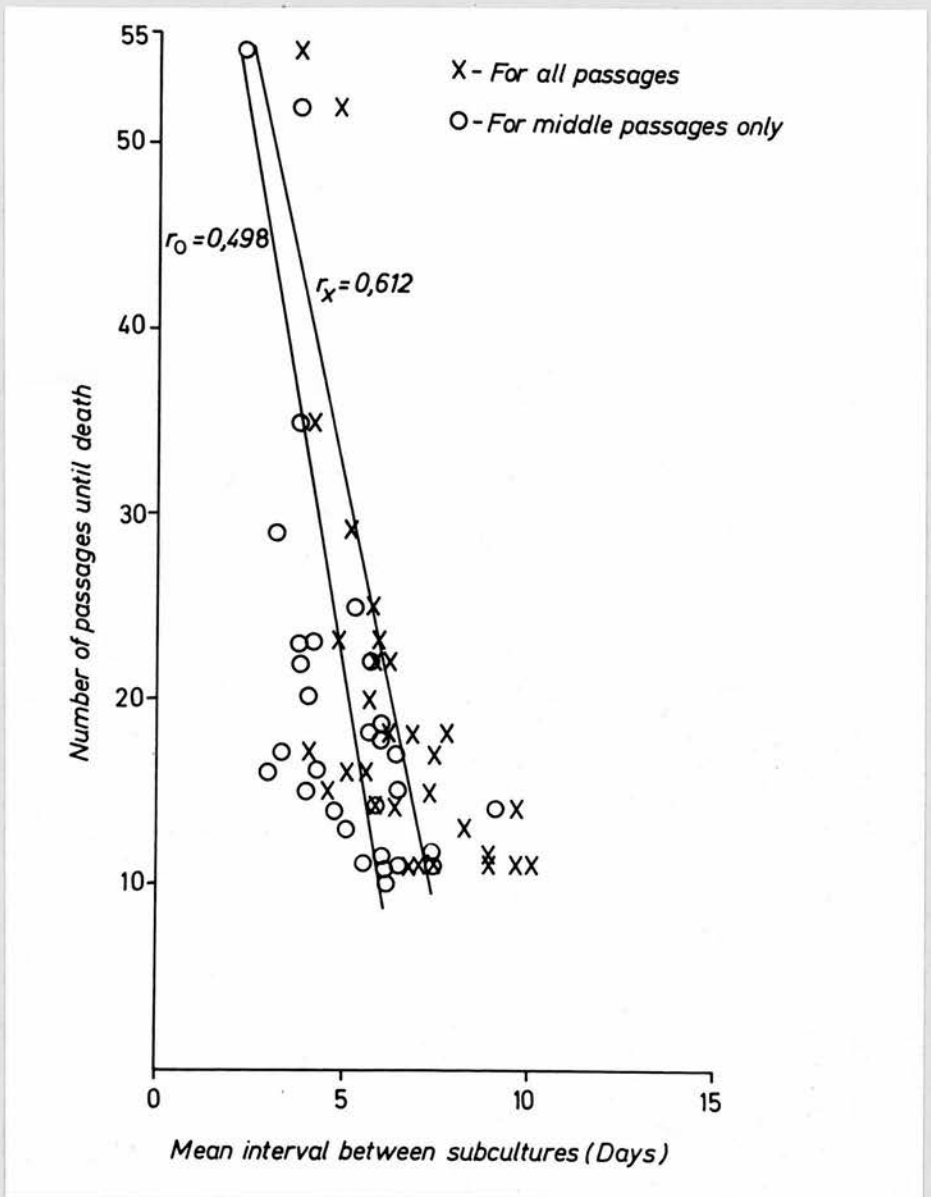


Fig. 3.4. Relationship between the number of passages for which a cell strain could be cultured prior to death and the mean interval between subcultures over all passages and over only the middle passages. Only cell strains which survived for more than 10 passages are included.

interval between subcultures was recalculated. The first two passages were excluded to allow for any lag phase and the last four to allow for the final slowing down. Only those cell strains which survived for more than 10 passages were considered, these comprised 30 cell strains

taken from both series. The reason for omitting those surviving for less than 10 passages was to allow the mean interval between subcultures to be estimated over at least five such intervals. The thirty cell strains showed the correlation between these two parameters on the original data ($r = -0.612$, $P < 0.001$). The adjusted mean interval values still showed the correlation ($r = -0.498$; $P < 0.01$) (Fig. 3.4).

The distribution of the number of passages for the two series is different. Although the means for each group are not significantly different the standard deviation in the second group is much greater. This is because three cell strains (176, 190 and 247) all lived much longer than the others. The distribution of the number of passages for each series and for the combined 49 cell strains are shown in table 3.5.

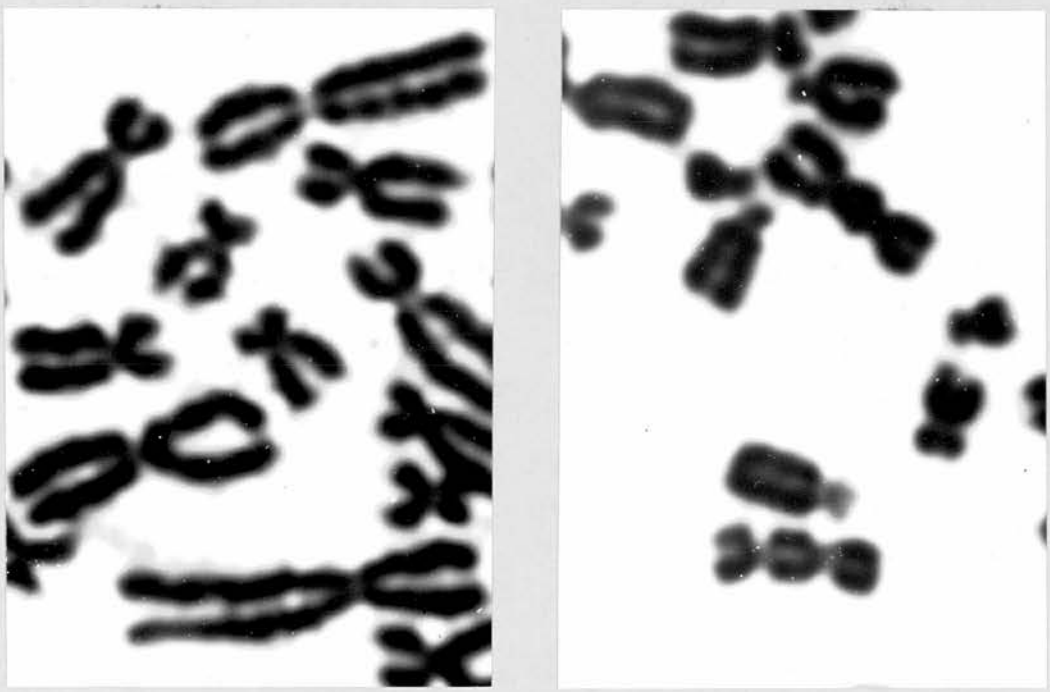
The serial samples of amniotic fluid collected from the four women yielded cell strains with properties as shown in table 3.6. These results from the second series add little to those of the first series. Again there is no more similarity within a group than there is between them and there are no trends within the groups

Table 3.5. Distribution of the number of passages for each series of cell strains and for the two series combined.

	No. of passages						
	0-4	5-8	9-12	13-16	17-20	21-24	> 25
Series 1	3	3	8	7	5	3	2
Series 2	2	6	4	2	-	1	3
Series 1+2	5	9	12	9	5	4	5

Table 3.6. Data on 9 amniotic fluid cell strains derived from 4 pregnancies.

Group	Laboratory No.	Gestation (weeks)	Days in 1° culture	No. of passages	Days as a cell strain	Mean sub-culture interval (days)
1	176	20	20	35	142	4.1
	189	23	28	9	84	9.3
	203	26	31	11	98	8.9
2	179	23	23	8	115	14.4
	195	26	41	8	72	9.0
3	191	20	43	2	25	12.5
	225	30	33	16	82	5.1
4	202	22	46	6	67	11.2
	206	26	55	8	71	8.9



a b

Fig. 3.5. Chromosome aberrations in senescent amniotic fluid cell strains; a - strain 176, 34th passage, cell showing 3? + chromosome; b - strain 247c, 49th passage, cell showing two dicentric chromosomes.

related to gestation.

Whilst a number of the cell strains in the second series were stored in the liquid nitrogen unit none were put back into culture and cultured to senescence.

The karyology of the second series was not monitored except for three of the longest lived cell strains (176, 190 and 247c) which were studied in more detail than those in the first series. The results of the chromosome analyses for these three cell strains are shown in table 3.7 and Fig. 3.5. It should be noted that the chromosome count is a centromere count, hence a dicentric is counted as two and an acentric is not counted. The quality of the chromosome preparations, both in terms of mitotic index and metaphase quality, declined as the cell strains approached death. Hence it was possible to score many more metaphases for ploidy than could be analysed. The three strains, especially 247c, showed an increase in structural aberrations in the last few passages prior to death. There appeared to be an increase in polyploidy as death approached but unfortunately there were too few mitoses in the late stages to establish this. Metaphases with less than 45 chromosomes were not included as these are most likely the result of artefact. The metaphases with 45 chromosomes are considered in table 3.8. If the expected number of chromosomes missing in each group is directly related to the number of chromosomes in each group then the findings in table 3.8 depart from expectation ($\chi^2_6 = 79.4$; $P < .001$). There is an overall deficiency of cells with chromosomes missing in all groups

Table 3.7. Cytogenetic data at various passages of three cell strains.

Strain	Passage	Chromosome counts					Abnormal cells
		45	46	47	Total	Polyploidy	
176	1°	1	10		11		45,XY,-C
	11		15		15	1/200	46,XY,+ace
	12	1	19		20	0/200	45,XY-C,+ace
	23		22		22	0/200	46,XY,F?+
	26	1	7		8	0/30	45,XY,-C
	34	3	12		15	1/120	46,XY,3?+; 45,XY,-G; 45,XY,-G,+ace; 45,XY,-2,+ace,+ace.
190	34	7	43		50	4/400	(45,XX,-F)x3; (45,XX,-G)x3; 45,XX,-C.
	39	4	45	1	50	1/300	(45,XX,-F)x3; (45,XX,-C)x2; 47,XX,+16.
	51	7	43		50	5/400	(45,XX,-F)x3; (45,XX,-E)x2; 45,XX,-C; 45,XX,-G.
	55	2	6		8	3/20	45,XX,-F; 45,XX,-G.
247c	1°	1	14		15		45,XY,-C
	20	2	48		50	6/200	45,XY,-G,45,XY,-C,+ace
	40	8	42		50	15/400	(45,XY,-G)x5; 45,XY,-C; 45,XY,-D; 45,XY,-G,+ace; 46,XY,-1,+C,+ace.
	47		2		2	2/11	
	49		3	1	4	0/5	47,XY,-C,-G,-16,+C, +dic,+dic. 46,XY,-G,-D,-B,+C, +dic.
	51	4	4		8	0/12	(45,XY,-G)x4; 46,XY,-G,-E,+dic.

Table 3.8. Distribution of the chromosomes missing from hypodiploid cells with 45 chromosomes for the three cell strains studied.

Strain	Chromosome Group							
	A	B	C	D	E	F	G	Total
176	1	-	3	-	-	-	2	6
190	-	-	4	-	2	9	5	20
247c	-	-	3	1	-	-	11	15
Total	1	-	10	1	2	9	18	41

except for F and G although this deficiency is probably not significant for the C group. The excess of F group chromosomes missing is most marked for strain 190 and the excess of G group missing is most noticeable for strain 247c.

The second series did not contribute anything new in the way of cell morphology which was not seen in the first series.

The first series had two cell strains excluded because of *in vitro* chromosome changes (table 3.1, page 59). These were found at the time of the first outbreak of mycoplasma contamination although these two cell strains did not yield mycoplasma on testing. In addition two urine cell strains showed chromosome abnormalities at this time and both of these strains yielded mycoplasma on testing. It is convenient to present the observations of *in vitro* chromosome changes in both amniotic fluid and urine cell strains in this chapter.

The first amniotic fluid cell strain to show a possible *in vitro* change was number 70. At the fourth

Table 3.9. Chromosome analyses for cell strain 115.

Passage	Karyotypes	
	46,XX (normal)	46,XX,Bp+
1°	17	-
11	40	-
20	2	38
23	-	20

passage 10 metaphases were analysed and all had a normal karyotype 46,XX. At the 22nd passage only eleven cells could be analysed but two of these had an extra F group chromosome. The cell strain died at the 25th passage and further chromosome preparations were unsuitable for analysis.

The second amniotic fluid cell strain to show an *in vitro* chromosome change was number 115. Chromosome results for this cell strain are shown in table 3.9. As can be seen from the table this strain was showing normal

Table 3.10. Chromosome analyses of urine cell strain 53.

Passage	Karyotype	
	46,XX (normal)	46,XX,t(2p-;16q+),inv(6)
1°	14	-
8	4	-
10	20	12
12	35	15
13	18	12
17	6	14
8/1F	30	-
10/1F	20	-

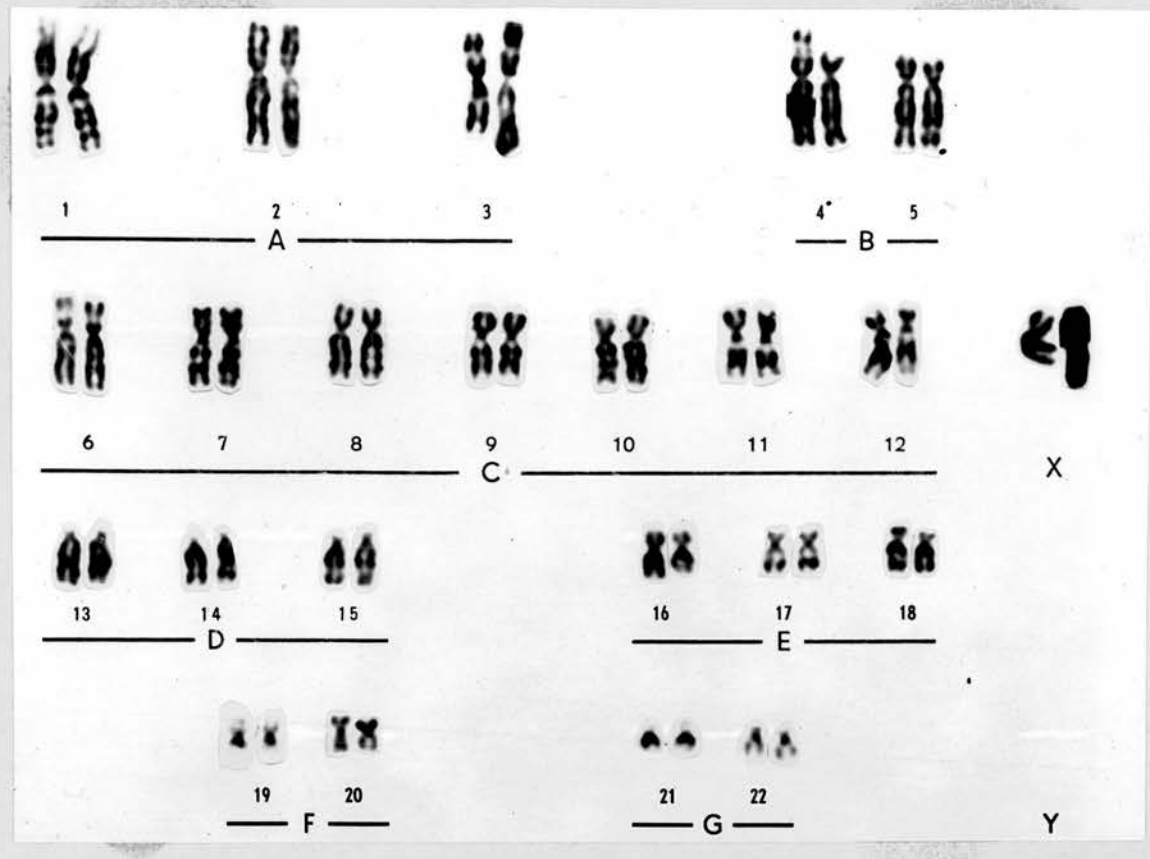


Fig. 3.6. Banded karyotype of cell from the 18th passage of culture of amniotic fluid cell strain 115. Note extra material on the short arm of chromosome number 4.

karyology at the eleventh passage, yet by the 20th passage most dividing cells had extra material on the short arm of chromosome number 4 (Fig. 3.6). The strain was discarded at the 28th passage due to suspected mycoplasma contamination.

The first urine cell strain to show in vitro chromosome changes was number 53. The results of chromosome analyses for this strain are shown in table 3.10. After the appearance of cells with abnormal karyotypes, cells from the strain which had been stored in the liquid nitrogen unit at the 5th passage were brought back into culture for chromosome studies. These showed only normal cells (passages 8/1F and 10/1F). The simplest

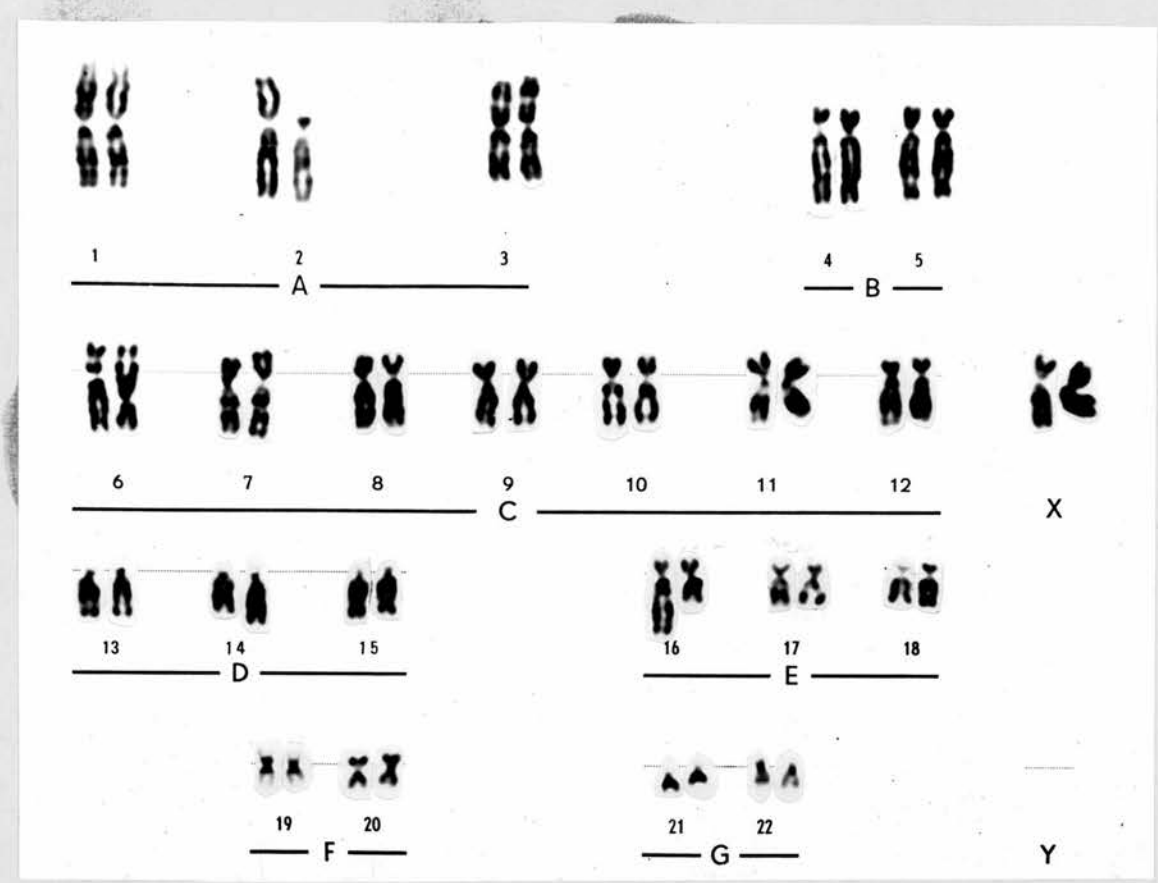


Fig. 3.7. Banded karyotype of cell from the 10th passage of culture of urine cell strain 53. Note translocation t(2;16)(p13;q24) and pericentric inversion inv(6)(p11q21).

explanation of these findings is that a clone of abnormal cells which carried an inversion and a translocation (Fig. 3.7) arose. This clone must have been at a selective advantage under the conditions of tissue culture and began to replace the normal cells. Mycoplasma arginini was recovered from the 16th passage and the cell strain was discarded in the 18th passage in a state of advanced senescence.

The most extensive chromosome changes were shown by urine cell strain 66. The results of initial chromosome studies are shown in table 3.11. After the 23rd passage most cells were very heteroploid, there were many

Table 3.11. Results of initial chromosome studies on urine cell strain 66.

Passage	Karyotype	
	46,XX (normal)	46,XX,2q+
4	26	-
8	45	5
13	20	16
15	7	23
23	-	11
9/1F	26	16
10/1F	2	8
11/1F	-	14

Fig. 3.8. Banded karyotype from the 13th passage of culture of urine cell strain 66. Note extra material on the short arm of chromosome number 2.

dicentrics, bizarre endoreduplication figures and cells with very large numbers of chromosomes (Figs. 3.8, 3.9, 3.10 and 3.11). This cell strain was reported to contain Mycoplasma arginini when first tested in the 21st passage and also from passage 19/1F. The mycoplasma contamination did not apparently inhibit cell growth and the strain was discarded in a state of senescence at the 35th passage. The presence of the initial chromosome abnormality in passage 9/1F indicates that this clone

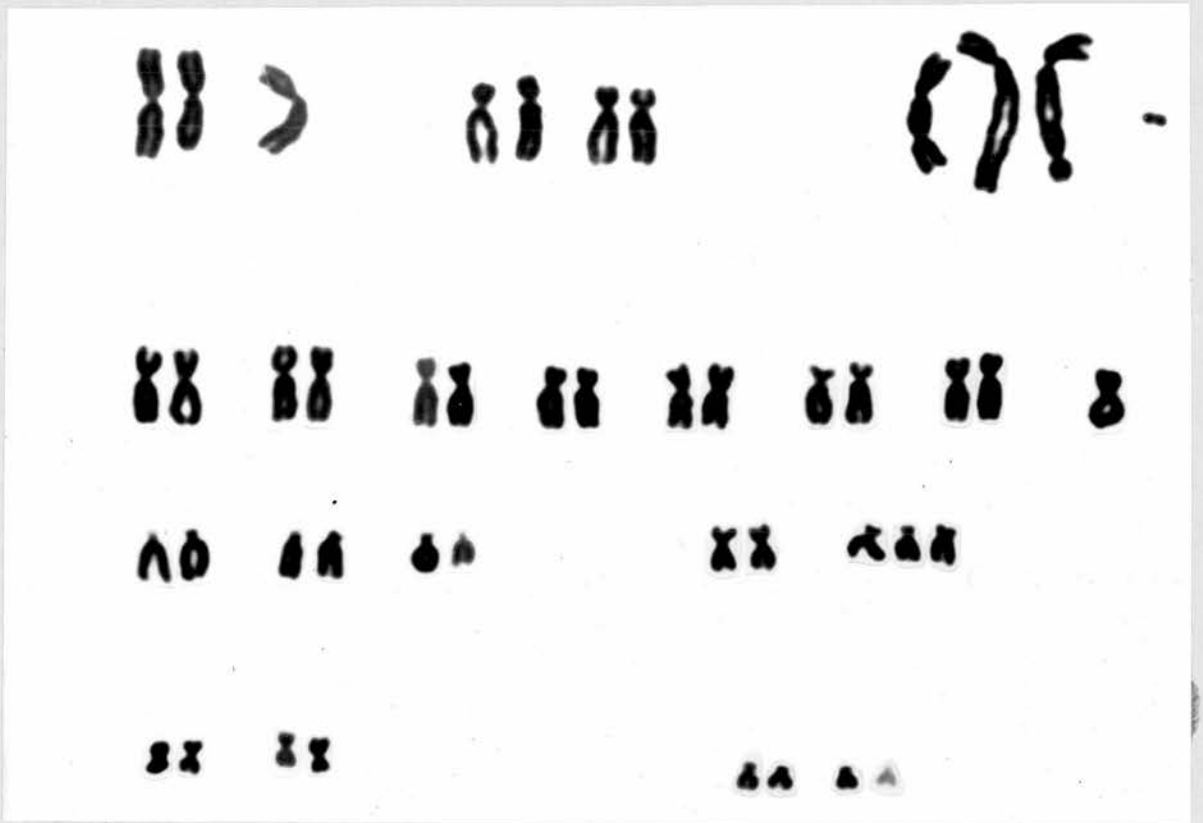
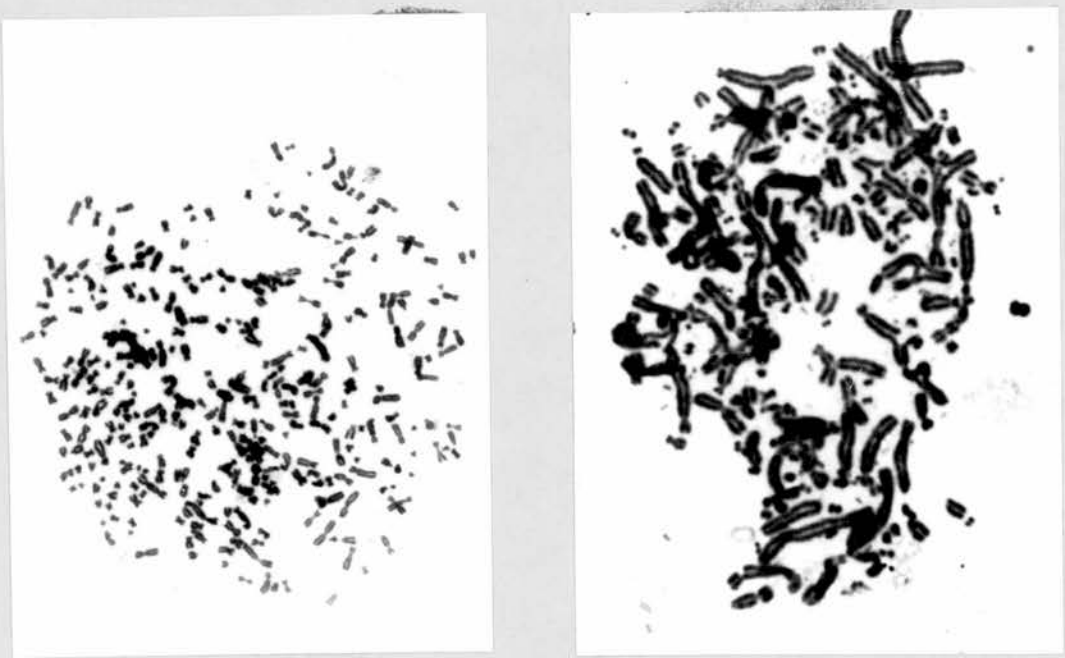


Fig. 3.9. Karyotype of a cell from the 27th passage of urine cell strain 66. Note apparent loss of three A-group chromosomes, one C-group chromosome and one E-group chromosome. Note presence of two dicentric chromosomes, small acentric fragment (minute) and large marker chromosome. This cell is representative of the early stages of heteroploidy shown by this cell strain.



(i) (ii)

Fig. 3.10. Two heteroploid metaphases from 31st passage of urine cell strain 66. Note (i) grossly heteroploid cell with high chromosome number and dicentric chromosomes and (ii) cell showing pulverisation of small chromosomes - this metaphase contains mainly long chromosomes with variable numbers of centromeres and minutes, there are only few intermediate-sized chromosomes.

arose prior to storage of the cell strain, at the 6th passage, in the liquid nitrogen unit.

The results of the second major phase of this study on amniotic fluid cell strains have been published in part (Sutherland and Bain, 1973, appendix III). This study of the time required to achieve an arbitrary quantity of cells for a hypothetical antenatal diagnosis of an inborn error of metabolism covered all amniotic fluids up to and including number 195, with the exception of those collected at induction of labour at term. This report (henceforth referred to as the first year's results) covered those amniotic fluids received in the period 1.12.71 to 30.11.72. The results will now be presented

Fig. 3.11. Portion of an endoreduplicated metaphase from the 31st passage of urine cell strain 66. Note bizarre chromosome configurations produced by chromatid exchange and number of "minutes".

for the second year, 1.12.72 to 30.11.73. All amniotic fluids received in this period are included except for those collected at induction of labour at term, those collected from anencephalic pregnancies and for numbers 293 and 286 which were not continued beyond the first passage. Hence the second year's results pertain to 52 amniotic fluids.

The origins of the amniotic fluids in the second year's results were different from those of the first year. There were no H.S. but there were specimens collected for diagnostic chromosome studies (D.C.S.), estimation of

α-fetoprotein levels (A.F.P.S.) and three specimens actually collected for the antenatal diagnosis of an inborn error of metabolism (D.B.S.). Data on the origin and cultural behaviour of the 52 amniotic fluids in the second year's study are shown in table 3.12 and Fig. 3.12.

There were some apparent differences in the times taken to achieve a biochemical result for the amniotic fluids of different origin. The Rh.S. were longer in primary culture and took longer to reach a biochemical result than did the other groups. These differences are however not significant statistically.

All cultures showed cellular proliferation as

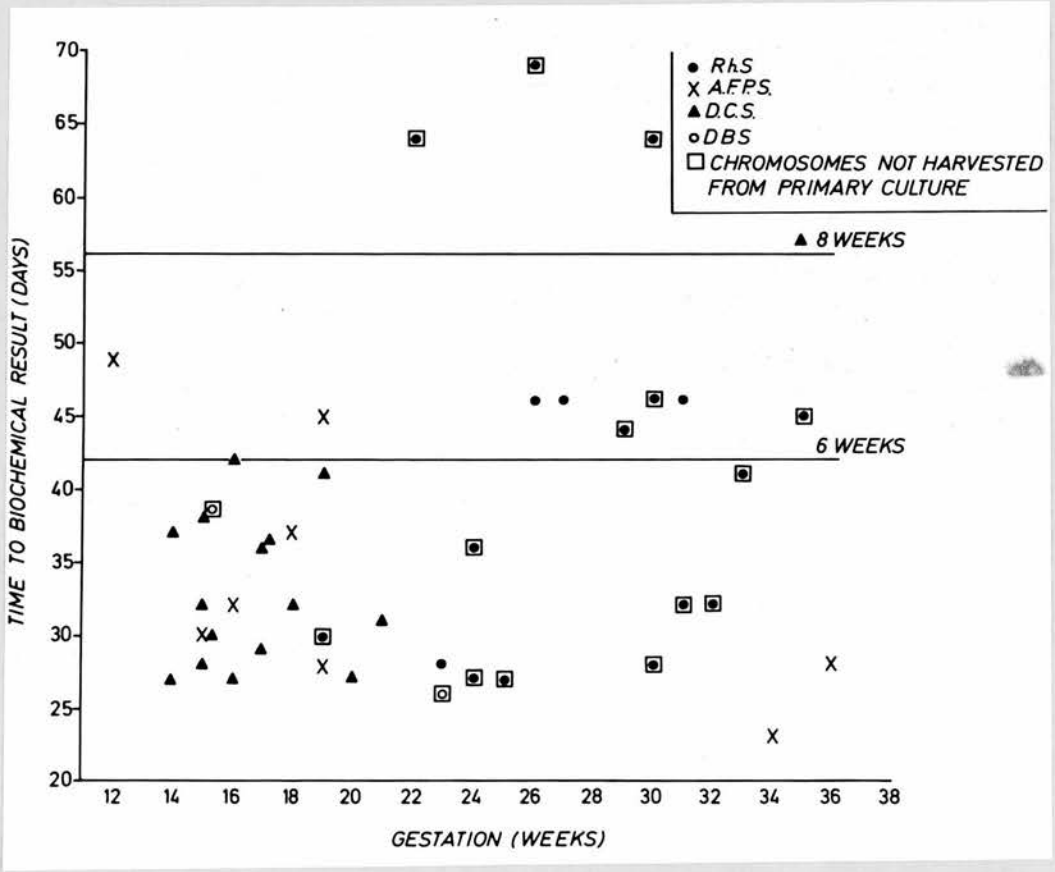


Fig. 3.12. Distribution of times taken to achieve sufficient cells for biochemical studies from the amniotic fluid cultures in the second year's study.

Table 3.12. Data on the origin and cultural behaviour of the amniotic fluids in the second year's study.

Origin	Number	Gestational age (weeks)			Number of biochemical failures	Days in Primary culture		Days to biochemical result		
		Mean	± S.D.	Range		Mean	± S.D.	Mean	± S.D.	Range
Rh.S.	20	27.90	± 4.14	19-35	2	29.44	± 9.80	41.72	± 13.21	27-29
D.C.S.	21	17.38	± 4.49	14-35	5	25.38	± 4.40	34.38	± 7.80	27-57
A.F.P.S.	8	21.13	± 8.89	12-36	-	24.25	± 6.27	34.00	± 9.01	23-49
D.B.S.	3	17.33	± 4.93	14-23	1	21 and 26		26 and 38		
Total	52	22.00	± 7.06	12-36	8	26.75	± 7.54	37.20	± 10.95	23-69

observed using the inverted microscope with the exception of one culture (214) which was lost to microbial contamination before it could be assessed. Two cultures failed to produce enough cells for a cytogenetic result (217 and 284). Hence the cytogenetic success rate would have been 49/52 if these studies had been required on all cultures. There were 5 primary cultures lost to microbial contamination after cytogenetic studies had been completed but before the primary culture could have been subcultured. No cell strains degenerated between the first subculture and the stage where there were enough cells for a biochemical result. If the time taken to produce enough cells for a biochemical assay was not a relevant factor, there were 44/52 successful cultures. If a time limit of 8 weeks was to be imposed then the success rate fell to 40/52 and if this was further reduced to 6 weeks then the success rate fell to 32/52 (61%). As in the first year's results the time taken to achieve a biochemical result was independent of gestational age (both within the various groups and for the series as a whole) and whether or not a coverslip had been removed from the culture for cytogenetic studies.

There were three rhesus iso-immunised pregnancies from which more than 3 serial samples had been collected and the data was available for analysis. These data are shown in table 3.13. The conclusions reached from the first year's results are supported by the second year's results. These results also demonstrate that, with one exception, the time in primary culture is responsible for

Table 3.13. Times required for biochemical and cytogenetic results and time in primary culture of amniotic fluid cell strains derived from serial samples of amniotic fluid collected from three pregnancies.

Pregnancy		Gestation	Days to biochemical result	Days in primary culture	Days to cytogenetic result
1		19	30	18	N
		30	64	26	N
		32	32	28	N
2	*	20	28	20	17
	*	23	41	28	21
		26	46	31	13
		29	43	36	N
		33	41	30	N
3	*	20	69	43	12
		27	46	30	12
		30	46	33	N

* from first year's results.
N cytogenetic preparations not made in the minimum time.

more than half the time required for a biochemical result. This feature of the results is also shown in table 3.12.

The essential features of the two series are compared in table 3.14. The differences between these two series are minimal. In spite of the loss of 6 primary cultures to microbial contamination in the second year, which resulted in a lower overall success rate, the success rate at 6 weeks was virtually identical for the two series. This was due in some part to the slightly shorter time required to reach results in the second year.

The results of the study of the effect of the level of serum supplement to the culture medium and the type of culture vessel used on behaviour of amniotic fluid cell strains are shown in table 3.15. The number of passages

Table 3.14. Comparison of the first and second years' results for the time to culture sufficient cells for a biochemical assay.

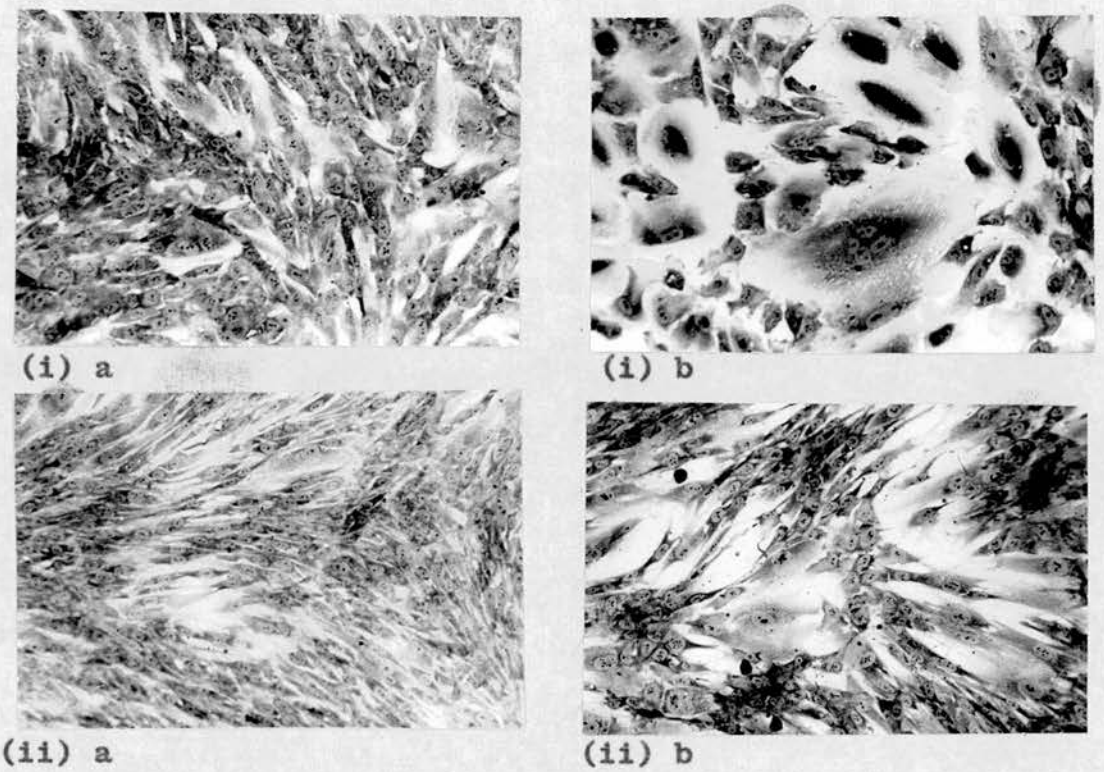
Parameter	First Year		Second year	
Number of amniotic fluids	62		52	
Mean gestational age (weeks)	24.2		22.0	
Number of failures	6		8	
Mean time to result (days)	41.4		37.2	
Overall success	56/62	(90%)	44/52	(85%)
Success at 8 weeks	49/62	(79%)	40/52	(77%)
Success at 6 weeks	36/62	(58%)	32/52	(61%)

is remarkably constant for each set of substrains despite the number of other variables. The type of culture vessel used appeared to be unrelated to cell strain behaviour. Although the mean interval between subculture was greatest for the substrains maintained in T-flasks the numbers involved are too small to attach significance to this. The shortest lived substrain in each set was cultured in a T-flask but, for 247, the longest lived was also cultured in a T-flask. The amount of serum supplement did not appear to affect any of the parameters examined. The one factor which did appear to have affected the number of passages was the primary culture from which the substrains were derived. In the case of 246, substrains a, c and e survived for a lower number of passages than those derived from the other primary culture. The reverse was true for 247.

Table 3.15. Results of long term culture of substrains of amniotic fluid cultures number 246 and 247.

Cell Strain	Sub-strain	% serum supplement in culture medium	Type of culture vessel*	Number of Passages	Days in 1° culture	Days as a cell strain	Mean subculture interval (days)
246	a	15	BB	18	28	107	5.9
	b	15	BB	22	26	123	5.6
	c	30	BB	22	28	138	6.3
	d	30	BB	24	26	140	5.8
	e	30	NTF	14	28	121	8.6
	f	30	NTF	22	26	149	6.8
247	a	15	BB	49	26	198	4.0
	b	15	BB	42	28	196	4.7
	c	30	BB	49	26	208	4.2
	d	30	BB	46	28	206	4.5
	e	30	NTF	52	26	248	4.8
	f	30	NTF	40	28	200	5.0

* BB - baby feeding bottle; NTF - Nunclon plastic T-flask.



(i) a (i) b

(ii) a (ii) b

Fig. 3.13. Flying coverslip preparations to show the effect of differential trypsinisation on cell strain morphology (Stain Giemsa, x 100).

(i) Substrain 246f in the 16th passage (a) of serial cultivation showing mainly epithelioid type III cells. The "trypsin resistant" substrain (b) 246f which has been in culture for the same length of time shows mostly type II cells including some which are multinucleate.

(ii) Substrain 247e in the 27th passage (a), and (b) "trypsin resistant" substrain in culture for the same period of time.

The effects of differential trypsinisation on the morphology of the substrains maintained in T-flasks is shown in Fig. 3.13. The effect on the two substrains of 247 was minimal. These were composed mainly of fibroblast-like cells from the early passages, but the "trypsin resistant" substrains retained a small number of epithelioid type II cells. The effects on the substrains of 246 were more marked. This strain started as a mixture of the three types of epithelioid cells. The

substrains serially subcultured lost the majority of the type II cells and eventually were composed mainly of type III cells. The "trypsin resistant" substrains however tended to do the opposite in that they were soon comprised of type II cells with few type III cells. This indicates that serial subculture can have a major effect on the morphology of amniotic fluid cell strains.

Unfortunately, some of the substrains of 264 became

Table 3.16. Data on the early behaviour of cell strains derived from fifteen primary cultures of amniotic fluid number 264.

Method of establishing primary culture[a]	Substrain	Days in 1° culture	Days to biochemical result
	a	43	-[c]
	b	-[b]	-
I	c	38	44
	d	36	44
	e	36	46
	f	25	32
	g	28	35
II	h	25	32
	i	22	32
	j	22	32
	k	22	35
	l	23	32
III	m	22	32
	n	22	32
	o	22	30

[a] - see chapter 2.

[b] - this primary culture degenerated and could not be subcultured.

[c] - this cell strain only survived one subculture.

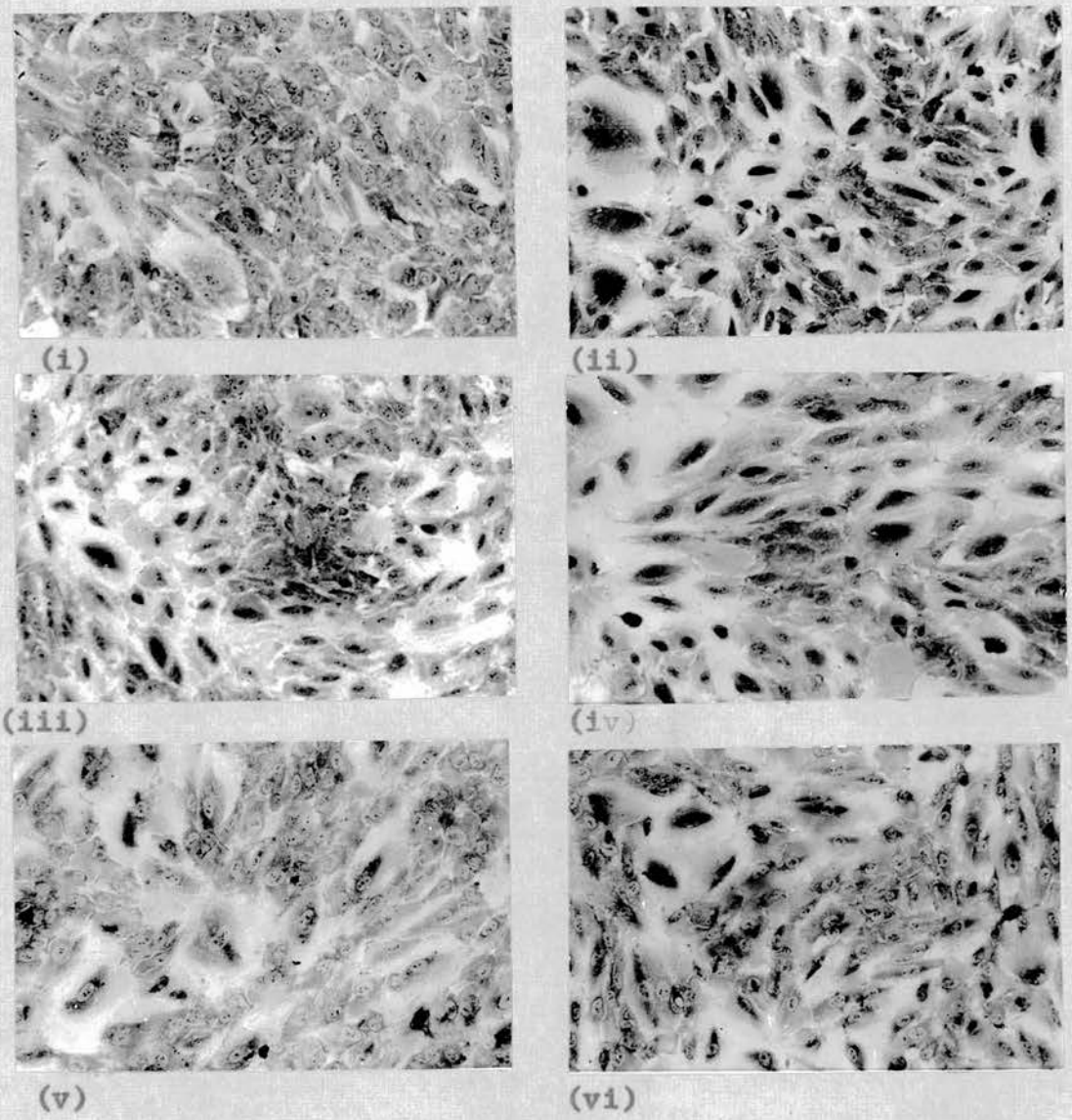


Fig. 3.14. Flying coverslip preparations showing the effects of the method of primary culture and of sub-culturing on cell strain morphology (Stain Giemsa, x 100).

(i) Second passage of substrain h, predominantly epithelioid type III cells.

(ii) Second passage of substrain i, there are considerable numbers of both type II and type III cells.

(iii) Second passage of substrain o.

(iv) Second passage of substrain e, predominantly type II cells.

(v) Tenth passage of substrain i, morphology is not changed from that in the second passage.

(vi) Tenth passage of substrain o, morphology is not different from that in the second passage.

contaminated with Mycoplasma hyorhinis hence the long-term culture results from this experiment are not valid. However, since this contamination did not occur until late in culture the results for the time to reach a biochemical result are valid as are some studies on cell morphology. The data on time required to achieve a biochemical result are shown in table 3.16. Method I was not as successful as methods II and III for producing amniotic fluid cell strains. It failed on two out of five occasions and when cell strains were produced the time taken was longer.

Examples of the morphology of the cell strains derived from primary cultures established by each of the methods are shown in Fig. 3.14. These strains were a mixture of epithelioid cells type II and III. Sub-culturing had little effect on the morphologies of these cell strains. It can be seen from Fig. 3.14 that the method used to establish the primary culture did not greatly affect the morphology of the cell strains derived from it.

DISCUSSION

Two phases of this work have already been discussed (Sutherland and Bain, 1973, Sutherland et al., 1974a, appendix III) and there is little to add to the individual aspects considered in these two papers except where additional data has been produced or other relevant work has been published.

The serial culture studies suffer from not having been carried out on cell strains derived from consecutive amniotic fluids. Although the first series of 31 strains came from 41 consecutive fluids, the exclusion of some cell strains because they were not cultured to senescence, for reasons such as microbial contamination or *in vitro* chromosome changes, introduces a degree of bias. Obviously the longer a cell strain survives the greater are the chances of it becoming contaminated. Even though the two cultures contaminated by bacteria were only in the first passage the others excluded due to mycoplasma contamination or chromosome changes were past the twentieth passage. Had these been included in the first series the mean number of passages and time as a cell strain would both have been greater. Hence, to a certain extent, the results for both series of cell strains cultured to senescence underestimate the growth potential of cultured amniotic fluid cell strains.

It is difficult to attribute biological significance to the statistical differences found between the two series. The cell strains in each series were in culture at different times, using different batches of culture

media and sera. The second series had 15% fetal calf serum supplement in the culture medium compared with 30% in the first series. It is tempting to attribute the statistical differences between the two series to this difference in serum supplement but such conclusion would not be valid. Todaro and Green (1964) found that adult skin fibroblast-like cell strains survived for a greater number of passages when the serum concentration was 30% than when it was 10%. Eagle (1973) found that the growth rate of human fibroblast-like cells increased with serum concentration over the range from 0.2 to 20%. The results of the present study do not support these findings for amniotic fluid cell strains but, because this effect of serum was not specifically tested in the two series, cannot be used as evidence against such an effect. However the experiment in which cell strains from two amniotic fluids (246 and 247) were grown in parallel in serum concentrations of 15 and 30% indicated that this factor does not influence the number of passages for which amniotic fluid cell strains can be cultured. This finding is in agreement with Litwin (1970) who found that increasing the serum concentration above 10% had no effect on growth rate or longevity of human fetal lung fibroblast-like cell strains.

An unexpected finding in both series of cell strains cultured to senescence is the highly significant correlation between the number of passages and the mean interval between subculture. This correlation was still significant when only those cell strains which survived

for more than 10 passages were considered and if the slower phases of growth, the initial and final passages, were excluded. The amniotic fluid cell strains showed the type of growth curve described by Hayflick and Moorhead (1961) for human diploid cell strains derived from fetal lung. It is possible that the correlation between growth rate and longevity found for amniotic fluid cell strains would also apply to diploid cells cultured from other tissues; indeed it does hold for urine cell strains (see chapter 7). There is no reference to such a correlation in the literature but Todaro and Green (1964) found that they could stimulate hampster fibroblasts to divide more rapidly and to survive a much greater number of passages before death by the addition of albumin to the culture medium. They found that human fibroblast-like cells survived for a greater number of passages but did not divide more rapidly under such conditions. Litwin (1970) studying human fetal lung fibroblast-like cell strains found that some " .... had a much slower growth rate and appeared to reach senescence earlier." He suggested that these may have been infected with some unascertained agent, however the present study indicated that the stage at which senescence is reached is continuous from degeneration in the primary culture to more than 50 passages. Miles (1964) had the impression that " .... more frequent passage enhanced the life span of cell strains ...." but was unable to quantitate this effect. It is not surprising that no other references

to this correlation have been made as there has been little work done on the serial cultivation of human diploid cell strains since that of Hayflick (1965). Even this work of Hayflick (1965) which fully established the limited *in vitro* lifespan of human diploid cells was based on studies of only 13 separate fetal cell strains. The biological basis of the correlation between lifespan in culture and rate of cell growth remains unknown and is worthy of further study.

The studies on cell strains derived from serial samples of amniotic fluid collected from Rhesus iso-immunised pregnancies in the second series add little to the corresponding work in the first series.

The main findings of relevance in the second series are the details of the karyology of the longest lived cell strains. These demonstrate that amniotic fluid cell strains can be regarded as diploid. The finding of dicentric and other structurally abnormal chromosomes in the senescent phases of the cultures is very similar to the findings of Saksela and Moorhead (1963) and Yoshida and Makino (1963) for human diploid cell strains of fetal origin. The distribution of the chromosomes missing from the hypodiploid cells is of interest. There is an excess of cells with F and G group chromosomes missing and a deficiency of cells with other chromosomes missing. Examination of the data of Yoshida and Makino (1963) revealed that very few hypomodal cells were found and the chromosomes missing from them were not identified. Analysis of the data of Saksela and Makino (1963) indicated

that the distribution of the chromosomes missing from the hypomodal cells accords with the expectation that the chances of any chromosome being absent are the same ($\chi^2_5 = 9.2$; N.S.). Rask-Madsen and Philip (1970) presented data on hypodiploid cells with 45 chromosomes found in cultures of human endometrium; the patterns of aneuploidy in these cells were similar to that found in the amniotic fluid cells in the present study. Court-Brown et al. (1966) found an excess of G and E 17/18 group chromosomes missing from hypodiploid cells from blood lymphocyte cultures. Hence the karyological findings in amniotic fluid cells indicate that the nature and quantity of aneuploid cells in this material are similar to those found in other human diploid cells in culture.

The in vitro chromosome changes seen in two amniotic fluid and two urine cell strains are of particular interest. These occurred at a time of mycoplasma contamination although mycoplasma was only identified positively in the urine cell strains. Chromosome abnormalities have been induced in cultured cells by infecting them with mycoplasmas (Fogh and Fogh, 1965; Aula and Nichols, 1967; Stanbridge et al., 1969; Kundsin et al., 1971). A variety of chromatid aberrations have been described along with an increased frequency of dicentrics and translocations. The study in which mycoplasma infected cultures of WI-38 were maintained and subcultured (Stanbridge et al., 1969) did not reveal the appearance of clones of chromosomally

abnormal cells. Kundsin et al. (1967) have claimed amnion cell cultures can be naturally infected with T strain mycoplasmas. If this is the case then it is conceivable that amniotic fluid could also be naturally infected with these organisms.

There are a number of other possible explanations of the *in vitro* chromosome changes not necessarily involving mycoplasmas. The cell populations which arose *in vitro* may not have been clones. The same abnormality may have been induced in a number of cells at the same time. This is unlikely as chromosome breakage is usually not at specific points on the chromosome although it has been shown that adenovirus type 12 can cause specific breakage of chromosome 17q?21 (Zur Hausen, 1967; McDougall, 1971). Hence it is possible that some agent capable of causing specific chromosome breakage was contaminating these cultures which showed *in vitro* chromosome changes. Another possibility is that amniotic fluid and urine cell strains may show chromosome instability (see chapter 4). Such an explanation is not very plausible but in the two amniotic fluid cell strains showing chromosome changes attempts to isolate mycoplasma were unsuccessful. *In vitro* chromosome changes, detected by the appearance of clones of abnormal cells, in skin fibroblast-like cell strains, were noted in a number of laboratories in the U.K. at the same time as those in the present series (Harnden, personal communication). These were not associated with mycoplasma and an unknown metabolite in the fetal calf serum used was suggested as a possible

cause. Most of the commercially available fetal calf serum in the U.K. comes from a single source.

The measurement of the times required to achieve a biochemical result provided data which was not available in the literature. The results of the second year added little to those of the first year other than to show that they were repeatable. One point which was perhaps not emphasised sufficiently in the discussion of the first year's results (Sutherland and Bain, 1973, appendix III) was that about two-thirds of the time delay was in the primary culture. This accentuates the need to develop microassay methods for enzymes involved in inborn errors of metabolism. There has been some progress in this direction. Jacoby et al. (1972) have developed a semimicroassay technique suitable for the antenatal diagnosis of argininosuccinic aciduria. Perhaps the ultimate in microassay techniques has been developed by Galjaard et al. (1972; 1973). These techniques make the assay of some lysosomal enzymes possible on a single cultured cell. In practice they can be used to perform the antenatal diagnosis of Pompe's disease within 7 to 10 days of amniocentesis using 70-250 cultured cells per assay. Such techniques will allow for the antenatal diagnosis of inborn errors of metabolism as quickly as, if not quicker than that of chromosome abnormalities.

The establishment of 13 cell strains from a single sample of amniotic fluid indicated that the time to a biochemical result can be influenced by the method used

to establish the primary culture; the method of Gray et al. (1971) being inferior to the other two methods used. Apart from the cultures in which the method of Gray et al. (1971) was used there was remarkable uniformity in the times required to produce enough cells for a biochemical result. Unfortunately this experiment could not be repeated because of the unavailability of large volumes of amniotic fluid.

The studies on the morphology of the cells in amniotic fluid cultures have been discussed in Sutherland et al. (1974a, appendix III). Results of further studies have shown how the process of subculturing can select against the epithelioid type II cells which have been termed "trypsin resistant". Melancon et al. (1971) separated epithelioid and fibroblast-like cells from amniotic fluid cultures of mixed cell types by using trypsin to selectively remove the fibroblast-like cells. This differential sensitivity to trypsin has been accentuated by the constant removal of the cells which could be readily detached with trypsin. The effect was more pronounced in the case of 246 than of 247 which initially was a more fibroblast-like cell strain.

Studies on the morphology of the cell strains of 264 indicated that this was not dependent on the method used to establish the primary culture. It is perhaps surprising that the cell strains developed from this sample were so similar in morphology since Gerbie et al. (1972) managed to produce cell strains of predominantly fibroblast-like and epithelioid morphologies from large

volumes of amniotic fluid. However they had initial volumes of fluid greater than the one in the present study and presumably set up more primary cultures.

This study of the serial cultivation of amniotic fluid cell strains has shown that there can be great similarities but also marked differences between these and other fetal fibroblast-like cell strains in growth characteristics, morphology, karyology and lifespan in tissue culture.

SUMMARY

This chapter on amniotic fluid cell strains in serial culture included studies of cell morphology, karyology, growth potential and time requirements for the antenatal diagnosis of inborn errors of metabolism. A correlation has been described between the growth potential and growth rate of amniotic fluid cell strains. The karyology of cell strains cultured to senescence has been monitored and an increase found in chromosome aberrations in senescent cultures. In vitro chromosome changes in two amniotic fluid and two urine cell strains are described and their possible origin discussed. The time required to produce an arbitrarily defined quantity of cells suitable for enzyme assay for the antenatal diagnosis of inborn errors of metabolism has been measured and it was found that although sufficient cells could usually be cultivated the time required was often too long for the results to be of practical value. Amniotic fluid samples collected at different times throughout individual rhesus-isoimmunised pregnancies yielded cell strains which were no more similar to each other in behaviour than to cell strains derived from different pregnancies. The type of culture vessel used and the serum concentration in the medium was found not to affect the lifespan of the cell strains. The behaviour of 13 cell strains cultured from a single amniotic fluid sample was studied. Three methods which were used to establish the primary cultures from which these cell strains were derived did not affect the morphology of the cell strains but one of the methods

was inferior for producing cells for biochemical studies. A classification of the cell types in amniotic fluid cultures has been proposed.

ADDENDUM

Since this chapter was completed the work of Greene et al. (1973) has become available. This is of relevance in that it is one of very few reports to present data on the behaviour of amniotic fluid cell strains. Although principally concerned with parameters affecting cryogenic storage these workers cultured four cell strains to senescence, which was reached between the 21st and 31st passage. If these cell strains were representative of the group studied then they would appear to have a greater average growth potential than those in the present series. One possible explanation for this difference is that Greene et al. (1973) cultured their cells in a medium different from that used in the present study. The four cell strains which they cultured to senescence were found to remain diploid, in agreement with present findings. The time delay before their primary cultures were ready for subculture averaged 11.7 days, much sooner than for the primary cultures in the present study wherein the average time in primary culture was about 27 days. The reasons for this marked difference are unknown; conditions of culture might be one factor but others could include differences in the initial cell inoculum into the culture vessel and the stage at which the primary cultures were subcultured since this is subjective and depends greatly on the operator.

Chapter 4

Cytogenetics of Amniotic Fluid

BACKGROUND

The development of human cytogenetics has recently been well summarised by Hamerton (1971). For many years there was controversy over the actual number of chromosomes in normal human cells. This was finally settled by Tjio and Levan (1956) who were the first to successfully apply the techniques of tissue culture to human cytogenetics. Clinical cytogenetics had to await the development of the simple leucocyte culture techniques using phytohaemagglutinin and the air-drying technique for preparing high quality metaphases. These techniques were put together by Moorhead et al. (1960) who described the first reliable method for preparing high quality metaphases from human leucocyte culture. Since this time, clinical cytogenetics has become a recognised discipline and chromosome analysis is available as a routine laboratory examination to most clinicians practising in the developed countries.

Little further technical progress in human cytogenetics was seen until the early 1970's when the identification of every chromosome in the human complement became possible. This began with the work of Caspersson et al. (1968) using fluorochromes to stain Chinese hampster and Vicia chromosomes. The more useful banding techniques were developed by Arrighi and Hsu (1970) who were the first to produce C-bands (see Paris Conference, 1971, for nomenclature). Their technique was modified by a number of authors to produce G-bands (e.g., Drets and Shaw, 1971; Sumner et al., 1971) which

enabled each chromosome to be readily identified.

The development of amniotic fluid cytogenetics has closely paralleled that of amniotic fluid cell culture as the initial reason for culturing amniotic fluid was to produce karyotypes. Klinger (1965) was the first to karyotype an amniotic fluid and the fact that this karyotype was male confirmed that he was indeed working with fetal material. Steele and Breg (1966) were the first to document chromosome analysis from amniotic fluid, at the time they recorded their work on the culture of amniotic fluid cells. The ability to karyotype amniotic fluid was rapidly confirmed by several groups (e.g., Thiede et al., 1966; Jacobson and Barter, 1967).

The methods for making preparations suitable for cytogenetic analysis have all used the same principles. These are the exposure of the cells in tissue culture to a mitotic arresting agent, the treatment of the cells arrested in metaphase by a hypotonic solution, and fixation of the swollen cells followed by air-drying and staining.

The technical approaches can be divided into two, according to whether the cells are harvested as a suspension or attached to the surface on which they are growing. Either method can be used for primary cultures or cell strains. However the harvest of primary cultures as a cell suspension can be difficult and unreliable with small cell populations. As a prompt result is required in diagnostic antenatal karyotyping, the best method is " .... that technic which gives the greatest degree of

success in the shortest amount of time ...." (Macintyre, 1971). This can almost always be achieved by harvesting primary cultures attached to the substrate (coverslip).

Regardless of which method is to be used, the first step is to produce cells arrested in metaphase. Many workers attempt to stimulate a wave of mitosis by sub-culturing or changing the tissue culture medium the day before harvesting. The metaphase arresting agent is added 4-6 hours before harvesting. Most workers use colchicine or colcemid for this purpose but some have used vinblastine (e.g., Thiede et al., 1966). The amount of colchicine (or colcemid) added varies about 500-fold. Most workers use a final concentration from 0.1 to 1 μg/ml. In some cases, however, this has ranged up to the excessive amounts used by Nelson (1973b) - 25 μg/ml., and by Valenti and Kehaty (1969) - 60 μg/ml. It is perhaps surprising that these workers were able to achieve success, especially as Fitzgerald and Brehaut (1970) have shown that 5-15 μg/ml. of colchicine depresses DNA synthesis and the mitotic index by increasing the length of the G_2 period in lymphocyte culture.

Most workers who have harvested their cells in suspension have used trypsin to remove them from the growth surface although some prefer pronase (e.g., Epstein et al. 1972).

A bewildering array of hypotonic treatments have been described. Sodium citrate has been used in concentrations ranging from 0.25% (Thiede et al., 1966) to 1.12% (Ferguson-Smith et al., 1971) for times ranging

from 10 to 45 minutes. Surprisingly the correlation between duration of hypotonic treatment and the concentration of sodium citrate appears to be small. Abbo and Zellweger (1970) used 0.7% sodium citrate for 45 minutes whereas Ferguson-Smith et al. (1971) used 1.12% citrate for 20 minutes. Other simple inorganic hypotonic salt solutions have included 0.3% NaCl (time unspecified) by Therkelsen et al. (1972) and 0.075 M KCl for 50 minutes by Nelson (1973[b]). Tissue culture medium diluted with water in varying proportions has also been used. Nadler and Gerbie (1972) used a solution of 1 part medium to 4 parts water for 30 minutes. Epstein et al. (1972) used only 2 parts medium to 5 parts water for 5 minutes. Gerbie et al. (1972) decided that epithelial-like and fibroblast-like cells required different hypotonic treatments. The fibroblast-like cells were treated with 1 part fetal calf serum to 5 parts water for 30 minutes, whereas the epithelial-like cells required the same treatment for 15 minutes followed by 15 minutes in a solution of 1 part fetal calf serum to 10 parts water. Most hypotonic solutions are used at 37°C. or when "warm".

The universal fixative for chromosome preparations made by air-drying is 1 part acetic acid to 3 parts absolute methanol. Some workers, however, then flood their slides with 45 or 50% acetic acid and dry them over a gentle flame (e.g., Gray et al., 1971; Gerbie et al., 1972, for fibroblast-like cells only). Most workers apparently transfer the cells, or coverslip, directly into the fixative. Ferguson-Smith et al. (1971,

and personal communication) gently waved the coverslip over the fixative before immersing it. Abbo and Zellweger (1970) and Nelson (1973b) add fixative to the hypotonic solution, hence gradually replacing the hypotonic solution by fixative. Most authors have used Giemsa as a stain for chromosomes although a significant minority have chosen acetic orcein.

MATERIALS AND METHODS

The amniotic fluids on which cytogenetic studies were carried out are those described in chapter II and appendix II table I, which were received over the two year period 1.12.71 to 30.11.73. Amniotic fluids which were collected for antenatal chromosome analysis over the additional period 1.12.73 to 28.2.74 are also included (appendix II, table III).

The method of making preparations suitable for chromosome analysis were as follows:

1. When a coverslip was seen to contain a suitable area of proliferating cells the medium in the Petri dish was changed.

2. The following morning the coverslip was removed from the Petri dish and placed in a tube of medium which contained 1 µg/ml. of colcemid.

3. About 5-6 hours later the medium was aspirated from the tube and replaced with 0.075 M KCl at room temperature and left for 5 minutes. (Early preparations were incubated at 37°C. but this was later found to be unnecessary.)

4. Fixation and staining proceeded as in Sutherland et al. (1973a , appendix III).

RESULTS

Chromosome studies were not carried out on all amniotic fluids. For practical purposes the fluids fell into two groups. The first comprised those fluids taken early enough in pregnancy for the chromosome result to influence the management of the pregnancy. Results were obtained from these specimens in the minimum possible time. The second group comprised those fluids in which chromosome studies were of little immediate clinical relevance because of gestational age or because the pregnancy had been terminated. Chromosome studies in this group were not necessarily carried out in the minimum time. Similarly, when there was more than one sample from any pregnancy such studies were not necessarily carried out on all samples.

Karyology of amniotic fluid samples

These results will be presented according to the reason for collection or source of the amniotic fluid. All results are based on examination of at least 10 metaphases.

Hysterotomy specimens: There were 20 amniotic fluids collected from hysterotomy specimens in this series. All these came from pregnancies terminated for social reasons, with one exception (62) which was at risk for maple-syrup-urine disease and where antenatal diagnosis had failed (see chapter 6). Chromosome results were achieved in 19 cases; there were 9 with normal male karyotypes and 10 with normal female karyotypes. The reason for failure in one case (43) was degeneration of the primary culture;

chromosome studies had been attempted but had failed. There was no opportunity to check the results of the chromosome studies with the sex of the fetus as this had not been recorded except for number 62 in which case there was no discrepancy.

Diagnostic α-fetoprotein samples: Details of the chromosome studies on the seven samples in this group which had normal α-fetoprotein levels are shown in table 2.1. With the exception of number 305 all these results were regarded as being of clinical significance. No abnormal karyotypes were detected.

Antenatal diagnosis of inborn error of metabolism: There were 5 samples collected from 4 pregnancies in this group.

Table 4.1. Cytogenetic results on amniotic fluid collected for α-fetoprotein assay and found to be normal in this regard.

Laboratory Number	Mother's Age (years)	Gestation (weeks)	Karyotype	Time (days)	Metaphases Counted
268	22	19	46,XX	14	12
276	25	16	46,XY	14	20
278	26	19	46,XX	12	25
280	23	18	46,XY	18	16
281	26	12	46,XX	24	20
305	26	34	46,XY	13	15
324	25	15	46,XX	16	20

Three produced normal female karyotypes and two cultures did not produce results due to degeneration. The sexes of the three infants at birth were as predicted.

Samples collected at induction of labour: There were 7 samples in this group. Three amniotic fluids showed normal male karyotypes and the other 4 fluids did not yield results because of failure to show growth in culture.

Rhesus specimens: The 61 specimens in this group were collected from 36 pregnancies and chromosome results were obtained for 33 of these. The reason for not obtaining results from three pregnancies was that one primary culture (214) and one culture at the first passage (64) was lost to microbial contamination and another culture (269) died before chromosome studies had been attempted. There were 18 normal male karyotypes and 13 normal female karyotypes. The sexes of the infants, checked from hospital records, were consistent with the predicted chromosomal sexes for 17 males and 9 females. One pregnancy had been terminated and the sex of the fetus not recorded, one pregnancy continues and three cases could not be traced. There were two abnormal karyotypes detected; one apparently balanced translocation (71), 46,XY,t(1,12)(p21;q21), (see Fig. 4.1) and one 47,XXX (207). Both these fetuses with a chromosome abnormality were stillborn. In the case of the translocation heterozygote the only relevant post-mortem finding was hydrops fetalis associated with Rhesus

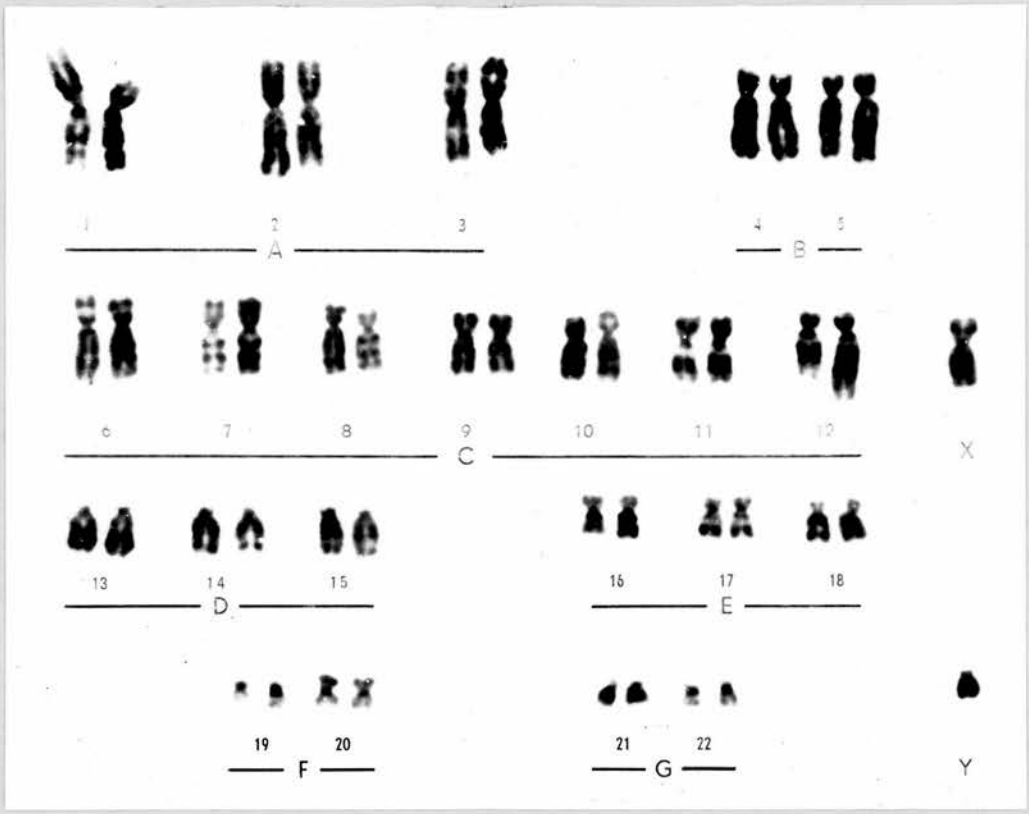


Fig. 4.1. Banded karyotype from amniotic fluid 71 showing apparently balanced 46,XY,t(1.12)(p21;q21).

iso-immunisation. The 47,XXX fetus was not affected by the Rhesus iso-immunisation and died from intrapartum anoxia. Of the 28 pregnancies on which chromosome studies had been completed records were available and the pregnancy had been concluded, there were four which resulted in stillbirths or neonatal deaths. Apart from the two with chromosome abnormalities, there was one infant with hydrocephalus and spina bifida who had died at a few days of age, and another infant, unaffected by Rhesus iso-immunisation who died from prematurity associated causes.

Cytogenetic diagnostic specimens: There were 33 amniotic fluids from 32 pregnancies in this group, all received over a period of two years and two months ending on 31.1.74 but with the majority (27) being received in the

last year of this period. All samples were collected from pregnancies of less than 21 weeks' gestation with one exception (200). This was from a woman aged 41 years at 35 weeks' gestation presenting with obstetric problems. Caesarian section was being contemplated but would not have been considered worthwhile if the fetus had been a mongol. Details of this and the other amniotic fluids in this group are in table III, appendix II.

One sample grew very slowly in culture and was considered to have failed (217) but fortunately amniocentesis was repeated and a result obtained. This was the only pregnancy in this series where the fetus had a chromosome abnormality (Gordon et al., 1974, appendix III). Another sample (220) grew very slowly in culture and after one week was considered to have failed. Repeated amniocentesis did not produce amniotic fluid but the original culture eventually yielded three metaphases after 38 days. A tentative diagnosis of normal female fetal karyotype was made; a normal female infant was born at term. One amniotic fluid was referred for sexing only since the fetus was at risk for X-linked muscular dystrophy. The fetus was diagnosed as male, the pregnancy terminated and the antenatal diagnosis of sex confirmed. All other cultures were unremarkable and the times taken to achieve cytogenetic results from the amniotic fluids in the sequence in which they were received are shown in Fig. 4.2. The times required to achieve results ranged from 7 to 38 days (mean 14.5 days). However after the first 6 cultures

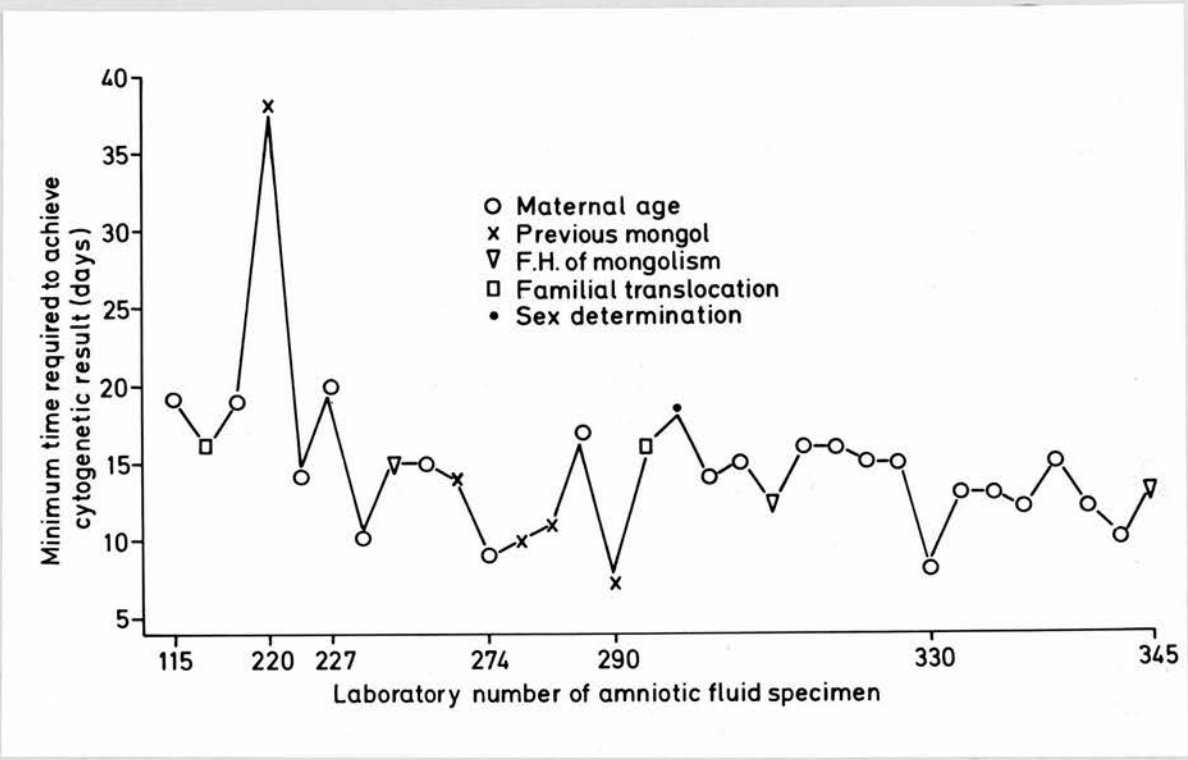


Fig. 4.2. Minimum times required to achieve cytogenetic results from amniotic fluid samples collected for antenatal chromosome analysis.

the mean time fell to 13.1 days and in no case was more than 18 days required.

Quality of Result

There are three factors which contribute to the quality of a chromosome result: the number of metaphases studied, the number of non-modal cells present and the quality of the metaphases available for study. This last factor is subjective; it relates as much to the skill of the cytogeneticist as to any absolute criterion, is difficult to quantify and will not be further considered.

All results for the non-diagnostic amniotic fluids were based on the examination of at least 10 metaphases. As these were of little clinical significance greater

numbers of metaphases were examined only if a chromosome abnormality was suspected from the first 10 cells. Similarly the number of non-modal and polyploid cells in this material were not studied in detail. Examination of the amount of aneuploidy and polyploidy has been carried out only on the cytogenetic diagnostic amniotic fluids.

The degree of polyploidy ranged from nil in 6 cultures up to about 18% (293), although in most cases it was less than 10%. Polyploidy was never a problem in reaching a cytogenetic diagnosis. Aneuploidy was mainly seen as hypodiploidy but occasional hyperploid and pseudodiploid cells were seen. The additional or missing chromosomes in the aneuploid cells are shown in table 4.2. Apart from these there was one cell in which there was a Dr and one cell, with missing D and C chromosomes, in which there was an acentric fragment present. No other structural aberrations were seen. The chromosome changes in the aneuploid cells appeared to be random in all cases.

The number of cells on which the result was based was small in some cases; six results were based on examination of less than 10 metaphases. In all these cases except 220

Table 4.2. Distribution of additional or missing chromosomes in aneuploid cells seen in primary cultures of diagnostic amniotic fluids.

	Chromosome group				
	C	D	E	F	G
Number missing	9	3	5	3	9
Number extra	3	-	-	1	-

further cells were examined so that eventually at least 20 cells were analysed. The chance of having to change a cytogenetic result after the initial group of cells had been studied was not regarded as great enough to warrant delay of the report as maternal anxiety regarding the outcome of the antenatal diagnosis was often considerable.

DISCUSSION

The number of chromosome abnormalities which have been detected in amniotic fluid is not great and the majority of these have been cases of Down's syndrome, either of regular or translocation karyotype (Milunsky, 1973). Very little attention has been paid to chromosome abnormalities present in amniotic fluid collected from pregnancies not normally considered to be at risk in this regard. Ferguson-Smith et al. (1971) karyotyped 17 fluids collected from hysterotomy specimens and all had normal karyotypes. Similarly Jacobson and Barter (1967) karyotyped 27 amniotic fluids collected at the time of therapeutic abortion, but from fetuses which were not at risk for chromosome abnormality, and these all had presumably normal karyotypes. Nadler (1968a) reported studies on 23 Rhesus iso-immunised amniotic fluids, those which were karyotyped were presumably normal. Mintzer and Reisman (1969) karyotyped 21 amniotic fluids and all were normal. Therkelsen et al. (1972) successfully karyotyped 41 out of 45 third trimester Rhesus iso-immunised fluids, all of which were normal. Chitham et al. (1973) found 3 chromosome abnormalities in 60 amniotic fluids, some, but not all, of which had been collected from pregnancies at risk for such abnormalities. The actual chromosome abnormalities were not identified nor was the source of the amniotic fluid in which they were found. Walker et al. (1971) diagnosed Klinefelter's syndrome by combined sex chromatin and quinacrine fluorescence studies of amniotic fluid cells from an iso-

immunised pregnancy of 33 weeks' gestation; this is the only report of a chromosome abnormality having been diagnosed from amniotic fluid collected from such pregnancies.

In the present series two abnormal karyotypes were detected amongst 33 Rhesus iso-immunised fluids and in both these cases perinatal death occurred. The significance of this finding is difficult to assess. The incidence of major chromosome abnormality in a sample of 11,000 unselected newborns studied in Edinburgh was 1 in 140 (Ratcliffe and Keay, 1973). On this basis the finding of 2 chromosome abnormalities from 33 fluids near term is about 8 times more than expected. However it is perhaps significant that the four perinatal deaths known to have occurred in this group included the two with chromosome abnormality. It has been shown (Sutherland et al., 1973, 1974; Machin, 1974; Bauld et al., 1974) that the incidence of chromosome abnormality in perinatal deaths is about 10 times higher than that in unselected neonates.

In the present series no other chromosome abnormalities were found in the low risk amniotic fluids. Several authors have reported finding chromosome abnormalities in amniotic fluids at risk for disorders not of chromosomal etiology. O'Brien et al. (1972) reported that a fetus at risk for Tay-Sachs disease was aborted because it had a chromosome abnormality although its N-acetyl-ß-D-glucosaminidase A level was normal. Similarly Ferguson-Smith et al. (1971) found that a male fetus aborted because of its risk of developing X-linked

granulomatous disease had Down's syndrome. Once amniotic fluid has been collected for any purpose in early pregnancy chromosome studies should be carried out to avoid the possibility that the fetus, although not having the disorder of prime concern, may be born with a chromosome abnormality.

Most series of amniotic fluid chromosome studies have been concerned with cases regarded as being at an increased risk for chromosome abnormalities. Some of the early series are summarised in Sutherland (1972 , appendix III) and the main recent series are compared with the findings in the present study in table 4.3. The findings in the present study are consistent with those found in the other series. It is not possible to pool the numbers in the various series and assess the incidence of chromosome abnormalities in fluids collected for a particular reason since the series of Milunsky (1973) contains some of the data of other series from the U.S.A. and Canada. However inspection of table 4.3 shows that two features of the early series summarised in Sutherland (1972 , appendix III) are no longer apparent. The recurrence rate for Down's syndrome in mothers who have previously had a child with this disease is much lower than the early series indicated. The number of unbalanced translocations produced by parents who are balanced translocation heterozygotes has fallen from the high level suggested by the early series but would still appear to be greater than the maximum recurrence rate of about 9% which epidemiological studies

Table 4.3. Recent series* of cases in which diagnostic antenatal chromosome studies have been performed.

Reason for Amniocentesis	Epstein et al. (1972)	Therkelsen et al. (1972)	Hsu et al. (1973)	Milunsky (1973)	Mulcahy and Jenkyn (1973)	Prescott et al. (1973)	Robinson et al. (1973)	Turnbull et al. (1973)	Present study
Previous child with Down's syndrome	23	5	62(1)	485(5)	4	23	42(1)**		6
Advanced Maternal Age > 35	9(1)	3	54(3)	255(4)	4(1)	2	55(1)	21(1)	2
> 40				347(9)	4	8			20(1)
Parental Translocation Carrier	1	6	2	93(17)		3(1)	5(1)	3(1)	1
Sexing only	5(4)	3(1)	6(3)	115(54)	1		10(2)	1	1(1)
Miscellaneous			44	188(1)	5	5	6	9	2
Total	38(5)	17(1)	168(7)	483(90)	18(1)	43(1)	118(5)	34(3)	32(2)

* All these series are not mutually exclusive, Milunsky (1973) contains accumulated data from most laboratories in the U.S.A. and Canada.

** Includes family member with trisomy as well as those with a previous child with Down's syndrome, one fetus was incorrectly diagnosed as tetraploid.

Figures in parentheses indicate number of chromosome abnormalities detected with the exception of balanced translocation heterozygotes and, where sexing was required, this is the number of males diagnosed.

had suggested (Hamerton, 1970).

Apart from Down's syndrome there have been a number of other chromosome abnormalities detected which are known to increase in frequency with advanced maternal age. These include Klinefelter's syndrome (Mulcahy and Jenkyn, 1973; Milunsky, 1973), Edward's syndrome (Hsu et al., 1973[a]; Turnbull et al., 1973; Gordon et al., 1974, appendix III) and Patau's syndrome (Butler et al., 1973). Other chromosome abnormalities unrelated to maternal age have also been detected, these include 47,XYY (Nadler and Gerbie, 1971) and 45,X (Pers. comm. to Nadler, 1971) as well as unbalanced reciprocal translocations (Butler and Reiss, 1970; Machin, 1974). The types of chromosome abnormalities which would be expected in advanced maternal age have been examined in Sutherland (1972 , appendix III).

Inspection of table 4.3 shows that the chances of finding a chromosome abnormality is greater than 1% regardless of the reason for the amniocentesis with the exception of the miscellaneous group. In this group more than 200 amniotic fluids have been karyotyped and only one abnormality found. This was a case of trisomy 22 which was detected in a woman who had previously had a child with the same abnormality (Milunsky, 1973). The probability of such a rare chromosome abnormality occurring spontaneously twice in one family must be extremely low and a parental chromosome abnormality, possibly mosaicism, must be strongly suspected. Hence it would appear that where the indications for antenatal chromosome studies are 'miscellaneous' there is in fact no genetic indication for

this procedure. The frequency of chromosome abnormalities detected in this group so far does not appear to be greater than would be expected in the general population. Apart from genetic considerations, other reasons such as maternal anxiety may well be valid for performing amniocentesis in these cases.

In the present series of diagnostic cases there has been only one chromosome abnormality detected, a case of trisomy 18 diagnosed from the amniotic fluid of a 41 year old mother (Gordon et al., 1974, appendix III). One fetus at risk for X-linked muscular dystrophy was diagnosed as male and the pregnancy terminated; the antenatal diagnosis was confirmed. A number of others have terminated pregnancies at risk for this condition. Riis and Fuchs (1966) monitored two at risk pregnancies and terminated one of them after a male fetus had been diagnosed.

The ultimate criterion of the quality of antenatal chromosome analyses is how often they coincide with the status of the fetus once this is known from conventional extrauterine studies. Since the majority of antenatal chromosome analyses indicate a normal fetus and are readily confirmed the best assessment of quality of results can be made from a study of mistakes which have been made. Sex has been misdiagnosed in a number of cases, in all instances a male fetus having been diagnosed as female - this has occurred at least 7 times (Milunsky, 1973). The most probable explanation for this mistake is that maternal cells rather than fetal cells have been

cultured. It would be expected that maternal cells would have been cultured in about an equal number of cases where the fetus was a female. This type of error would go undetected unless the fetus had a chromosome abnormality which would not be diagnosed until birth. Hence it would appear from the data of Milunsky (1973) and Nadler (1970) that in about 1% of cases maternal cells will be cultured rather than fetal cells. Harvesting chromosome preparations from more than one culture may help to overcome this but there is no data available on this point. Macintyre (1971) reported that 1 culture out of 7 grown from a single sample of amniotic fluid contained a proportion of female cells, all the other cultures contained only male cells.

Apart from the misdiagnosis of sex few other errors have occurred. Kohn and Robinson (1970) terminated a pregnancy on the grounds of total tetraploidy in an amniotic fluid culture; in the light of subsequent experience since this should not happen again. Most amniotic fluid cultures contain a proportion of tetraploid cells; this was always less than about 15% in the present series. Walker et al. (1970) reported that 23% of 110 amniotic fluid cultures contained more than 10% polyploids but only 7% had more than 30% polyploids and 3 of these contained only polyploid cells. In 2 of these 3 cases other fetal material studied showed that the fetuses were diploid. Hence tetraploidy in cultured amniotic fluid cells is normal and not an indication of fetal tetraploidy which, although extremely

rare, would probably be undiagnosable at present. Apparently polyploid cells should always be checked to ensure that they are not triploid since triploid fetuses are more common than tetraploids and triploid cells are not normally found in amniotic fluid cultures.

A number of authors have discussed the problem of aneuploidy in cells from normal individuals (see chapter 3). In the present study the degree of aneuploidy was low and did not cause concern regarding the fetal karyotype. Any discussion of the problem of aneuploidy cannot be divorced from the problem of the diagnosis of mosaicism. A number of antenatal diagnoses are based on examination of a small number of metaphases, as few as 3 in the present series and 4 in the series of Ferguson-Smith et al. (1971). Others have counted many cells, for example Mulcahy and Jenkyn (1973) examined 112 cells from a single case. Nevertheless, mosaicism has rarely been reported and when this has occurred fetal studies have indicated that the diagnosis of mosaicism was wrong. Hsu et al. (1973) and Kardon et al. (1972 a,b) all recorded the same case of an alleged XO/XY mosaic. Their first sample of amniotic fluid contained only XO cells and the second only XY. They further suggested that the XO cells had translocation of Yq onto Bp but the argument in favour of this was most unconvincing. The pregnancy was terminated and resulted in a male fetus with no detectable abnormalities. The explanation of these findings is not clear but it is probable that misdiagnosis of mosaicism occurred. Lee

et al. (1972) reported finding one cell in 50, in a diagnostic amniotic fluid, which had an extra G group chromosome, the pregnancy was allowed to continue and a normal infant resulted. This is more a problem of the interpretation of a single aneuploid cell than a diagnosis of mosaicism. Kajii (1971) reported finding four out of 64 cells from the amniotic fluid of a 15 week fetus, collected at hysterotomy, with an extra C group chromosome. Cytogenetic studies on cells cultured from other fetal tissues had normal karyotypes. Kajii (1971) suggested that a cell line with an extra C group chromosome had arisen *in vitro*. Hence misdiagnosis of fetal mosaicism had again occurred from studies on cells cultured from amniotic fluid.

Hsu et al. (1973) reported two cases which might be regarded as mosaics. In one case, after treatment of a culture with amphotericin for a fungal infection, 8 out of 10 cells had karyotype 46,XX,Dq-. In the second case 3 out of 27 cells had a karyotype 46,XY,+D,-G with the remaining cells being normal. Both infants were presumably normal although chromosome studies were done on only the latter. These two cases which were considered by Hsu et al. (1973) to be due to *in vitro* chromosome changes, together with that of Kajii (1971) are qualitatively very similar to the chromosome changes noted in two amniotic fluid and two urine cell strains in the present series (see chapter 3). Do amniotic fluid cells in culture have a tendency towards karyotype instability? Extensive cytogenetic studies on a number

of cell strains throughout culture to senescence, in the absence of antibiotics and with constant screening for viruses, mycoplasma, etc., would be required to answer this question. On the other hand these may not be *in vitro* changes. The number of colonies in an amniotic fluid culture is small and if one of the colony forming cells had a chromosome abnormality this could explain the findings. There is some evidence (Uhlendorf, 1970) from the autoradiography of primary female amniotic fluid cultures heterozygous for the Lesch-Nyhan syndrome that the colonies in these cultures are actually clones.

In spite of the few errors which have been made in antenatal cytogenetic studies it is worth remembering that no case has been recorded of an infant being born with an unsuspected chromosome abnormality. This is however little cause for complacency and continuous attention must be paid to the quality of the results of antenatal cytogenetic analysis. The criteria suggested by Hamerton (1971) for the diagnosis of mosaicism cannot be fully applied to amniotic fluid cytogenetics and such diagnosis in this field is very difficult. There can be little doubt that before a pregnancy was to be terminated on the grounds of fetal mosaicism that amniocentesis should be repeated. In the light of reported experience any diagnosis of mosaicism should be regarded with considerable scepticism until confirmed by extrauterine studies.

The present study of amniotic fluid cytogenetics has, with other similar studies in the literature,

demonstrated that amniotic fluid culture is a simple, reliable and rapid means of establishing the fetal karyotype. This technique is particularly useful for screening pregnancies at high to medium risk of having a chromosome abnormality and, now that its reliability has been established, can be extended to selective population screening (Sutherland, 1972; Bain and Sutherland, 1972, appendix III) as a programme of preventive medicine once the obstetric safety factors have been fully ascertained.

SUMMARY

Cytogenetic studies have been carried out on some amniotic fluids not normally regarded as being at risk for chromosome abnormality. Two cases of chromosome abnormality were found in 33 Rhesus iso-immunised pregnancies and the significance of this finding is discussed. A series of 32 diagnostic cytogenetic antenatal assessments were performed, one fetus with trisomy 18 was detected and the pregnancy was terminated. The quality of cytogenetic results from amniotic fluid is considered; findings of polyploidy and mosaicism are discussed along with errors which have occurred in antenatal diagnosis. It is concluded that antenatal chromosome analysis is applicable to selective population screening for the purpose of reducing the incidence of chromosome abnormalities in the population.

Chapter 5

Amniotic Fluid Macrophages

INTRODUCTION

In 1972 Brock and Sutcliffe reported that amniotic fluid from a retrospectively studied series of amniotic fluids collected from anencephalic pregnancies had elevated levels of α-fetoprotein. The measurement of α-fetoprotein in amniotic fluid has subsequently become routine for the antenatal diagnosis of anencephaly and, probably, other defects of neural tube closure (see Sutherland et al., 1973b, appendix III for references).

Since amniotic fluids are usually collected for α-fetoprotein estimation early in the second trimester of pregnancy, any other information which can be gained on the condition of the fetus is of value. Hence the cells from such specimens of amniotic fluid were cultured for chromosome studies. Amniotic fluids from two cases of anencephaly were found to contain large numbers of cells which appeared to be fetal macrophages (Sutherland et al., 1973b, appendix III). As the presence of fetal macrophages in amniotic fluid had not been previously described, further studies were clearly indicated.

The macrophages associated with the placenta are known as Hofbauer cells (Hofbauer, 1903). These macrophages have been shown by sex chromatin studies to be of fetal origin (Bourne, 1962). In a cytological study, Huisjes (1968) found no abnormal cells in the amniotic fluid from two cases of anencephaly and one of spina bifida although he did not recognise macrophages in normal amniotic fluids as did Casadei et al. (1973). Fox (1967) has shown that Hofbauer cells are increased in number in

the placental villi from pregnancies complicated by Rhesus iso-immunisation and diabetes mellitus. These observations raise the following questions. Do the increased numbers of Hofbauer cells seen in these pregnancies show up as an increase in amniotic fluid macrophages? Are the macrophages in Rhesus amniotic fluid due to the same factor which causes an increase in Hofbauer cells?

One possible source of the macrophages in anencephalic amniotic fluid is the fetal lesion. Exposure of highly vascularised tissue, with only a membrane between the fetal circulation and the amniotic fluid, might allow blood macrophages to enter the amniotic fluid. Free communication between the cerebro-spinal fluid (C.S.F.) and the amniotic fluid might allow macrophages from the C.S.F. to enter the amniotic fluid. Wollard (1924) claimed that under the influence of irritants the mesothelial cells of the arachnoid and pia mater are set free into the C.S.F. as macrophages. Greenfield and Carmichael (1925) stated that macrophages were present in the C.S.F. in cases of sub-arachnoid haemorrhage, tumours and tuberculous meningitis but " .... have no special pathological significance". Chester et al., (1971) have reported the presence of macrophages in C.S.F. from cases of hydrocephalus.

Two lines of approach to this study of macrophages in amniotic fluid were made. The first, and most clinically relevant, was to ascertain whether the observation made in two cases was applicable in general.

Is an increased number of macrophages in amniotic fluid diagnostic of anencephaly and possibly other open neural tube defects? The second approach was to study the nature of the macrophages and to try and obtain evidence of their origin.

MATERIALS AND METHODS

Samples of amniotic fluid from normal and abnormal pregnancies and C.S.F. obtained at autopsy from hydrocephalics were studied for comparison with amniotic fluid from pregnancies in which the fetus was either known or suspected of having a gross C.N.S. defect.

The macrophage content of the amniotic fluids was quantitated. Primary amniotic fluid culture was set up as outlined in chapter 1. A coverslip was removed from the Petri dish 20 hours after the culture had been set up, washed twice in P.B.S., fixed in three changes of absolute methanol and stained with Giemsa. The number of cells adhering to the coverslip was counted in 10 randomly chosen low power (x100) fields. The count was adjusted for the volume of amniotic fluid which had been used to establish the culture and the glass adherent cell count was then expressed as the number/10 low power fields/10 ml. of amniotic fluid. If this count is multiplied by 6.25 then the result is the macrophage count/ml. of amniotic fluid - the area of a low power field is $\pi(1)^2$ mm^2 and the area of the Petri dish is $\pi(25)^2$ mm^2 hence the area of a low power field is 1/625th of the total area of the Petri dish. The assumptions made in using this parameter, that the attached cells are evenly distributed on the substrate, that the degree of attachment is independent of the cell innoculum and that all the attached cells are macrophages, are probably not correct. Nevertheless, the resultant count is useful for comparing amniotic

fluids.

Initial erythrophagocytosis experiments were carried out using sheep red blood cells sensitised with rabbit haemolysin (Sutherland et al., 1973b, appendix III). In further experiments human red blood cells sensitised with rabbit anti-human red cell serum according to Habeshaw (1970) were used. Phagocytosis experiments were carried out by placing the coverslip containing the macrophages in a 30 mm. Petri dish, adding one ml. of tissue culture medium (Ham's F_{10} with 30% fetal calf serum) and one ml. of a 5% suspension of the red blood cells in serum free Ham's F10 medium. The culture was incubated for two hours at 37 C in an atmosphere of 5% CO_2 in air. The coverslip was then washed, fixed and stained with Giemsa as outlined above.

RESULTS

Macrophage content of amniotic fluid.

Throughout this chapter the glass adherent cell count after 20 hours in culture (number/ 10 low power fields/ 10 ml. of amniotic fluid) will be used synonomously with macrophage count, although all the glass adherent cells are probably not macrophages. Macrophage counts were carried out on amniotic fluids from normal and abnormal pregnancies and the data are given in table IV, appendix II and summarised in table 5.1 and Fig. 5.1.

Table 5.1. Macrophage counts in amniotic fluids from normal and abnormal pregnancies.

Nature of fluid	Number*	Gestation* (weeks)	Macrophage* count
Normal	41	17.9[a] (14-38)[b]	11.8[a] (0-41)[b]
Rhesus iso-immunised	7	27.6 (19-35)	101 (1-276)
Hydramnios	4	29.8 (20-36)	8.3 (1-17)
Anencephalic	6	25.2 (15-36)	1704 (576-6226)
Spina bifida	2	13 and 14	82 and 168
Diabetic	1	38	14
Other	2	18 and 22	445 and 232

* Where more than one sample from any pregnancy was studied only the first one received was included.

[a] mean.

[b] range.

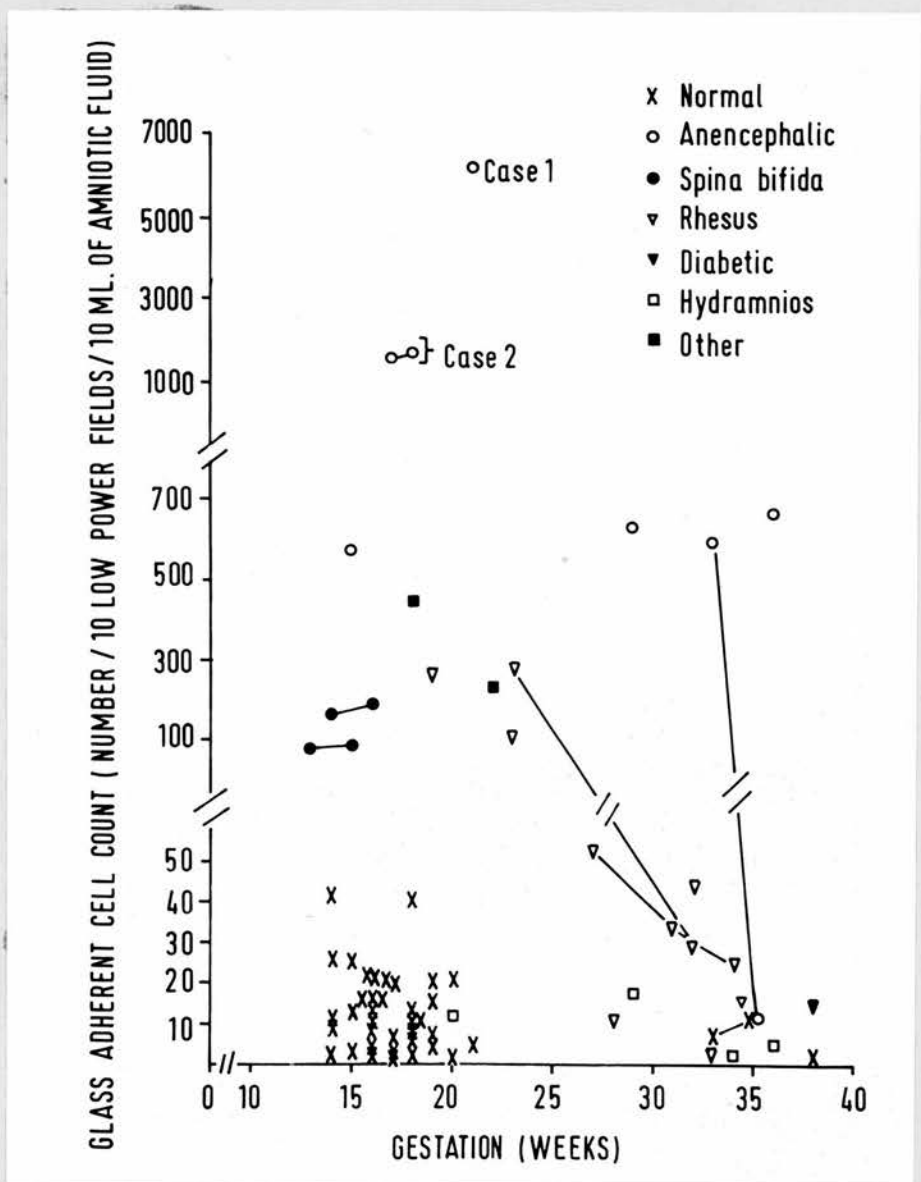


Fig. 5.1. Macrophage counts of all amniotic fluids included in this study in relation to gestation. Case 1 and case 2 refer to the two anencephalic amniotic fluids in Sutherland et al. (1974, appendix III).

The amniotic fluids in each category will be considered separately:

Normals: The macrophage counts in the normal amniotic fluids ranged from 0 to 41, but in only two samples were the counts above 25. The macrophage counts did not appear to change with gestation but this can only be confidently concluded for the range from 14 to 20 weeks.

There was no correlation between the total cell innoculum, which ranged from less than 10,000 cells up to 1.1 x 10^6 cells per Petri dish, and the macrophage counts. There was no correlation between the α-fetoprotein levels and the macrophage counts.

Rhesus iso-immunised fluids: The macrophage counts in these fluids ranged from 1 to 276. There appeared to be a fall in the count with increasing gestational age although the numbers studied were too small for this to be significant. The macrophage counts in some of these

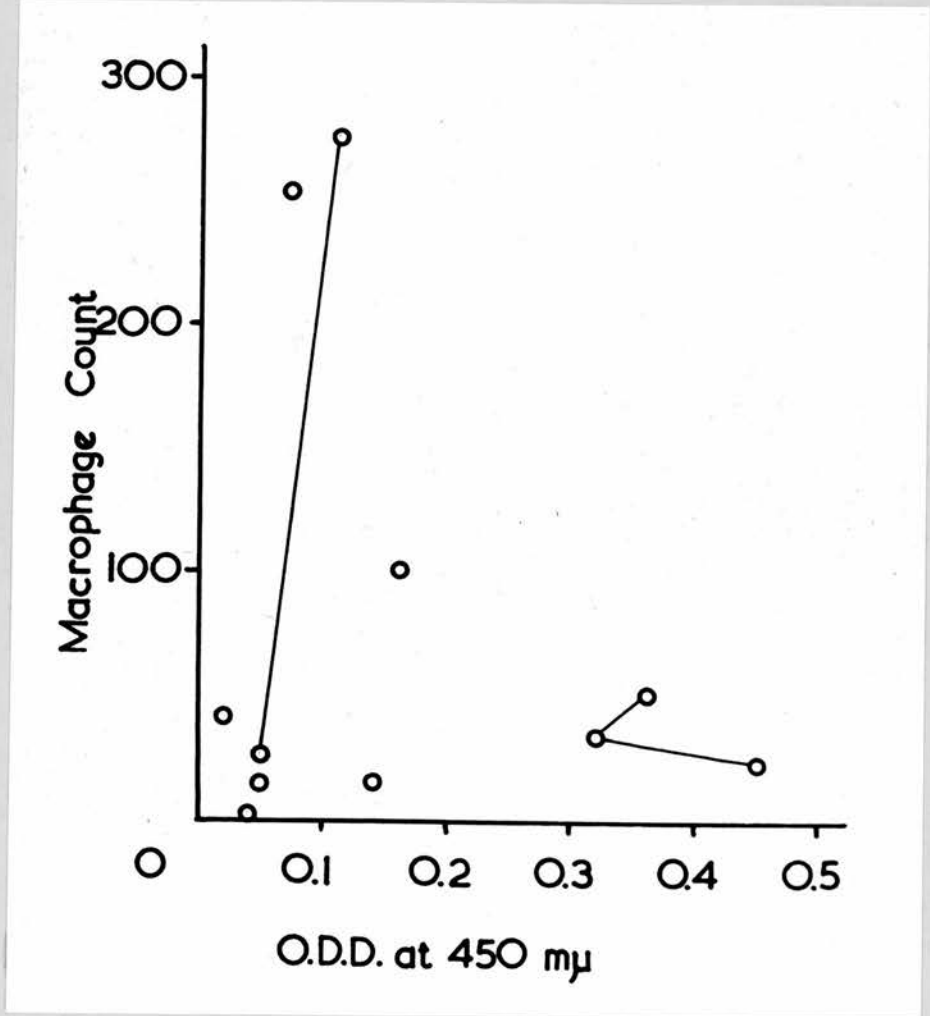


Fig. 5.2. Relationship between macrophage counts and O.D.D. at 450 mμ for Rhesus iso-immunised amniotic fluids.

fluids were much higher than in the normals although the range was very wide. There was no relationship between the macrophage counts and the O.D.D. at 450 mμ (Fig. 5.2). In the cases where more than one sample of amniotic fluid was collected from a single pregnancy the macrophage counts varied considerably.

Hydramnios amniotic fluids: There were only four fluids from such cases and in these the macrophage counts were all within the same range as for the normals. There were no fetal abnormalities in this group.

Anencephalic amniotic fluids: There were 8 fluids from 6 pregnancies in this group. If only the first sample from each pregnancy was considered, the macrophage counts ranged from 576 to 6,226 (Fig. 5.3). These counts were all well above those from the normal fluids

Fig. 5.3. Glass adherent cells from anencephalic amniotic fluid (273) after 20 hours in culture. (Giemsa, x100).

Table 5.2. Data on amniotic fluids from the twin pregnancy in which one twin was anencephalic.

Fluid No.	Gestation (weeks)	α-fetoprotein (μg/ml)	Macrophage count	Chromosomal sex
Twin I (304)	33	26	590	♀
Twin II (305)	33	1	6	♂
Twin I (307)	35	21	10	♀
Twin II (308)	35	3.5	10	♂

and do not overlap those from the Rhesus iso-immunised fluids. In the first case in which more than one sample was available from an anencephalic pregnancy (case 2, Sutherland et al., 1973b, appendix III) there was close agreement between the counts. In the second such case the findings were ambiguous. This was from a twin pregnancy in which there was a normal male twin and an anencephalic female twin. Some data on the fluids from this pregnancy are shown in table 5.2. The reasons for the failure to find a high macrophage count in the second sample from the anencephalic twin are unknown. The possibility that the samples from the two sacs were confused is remote but cannot be completely eliminated. This was the only anencephalic amniotic fluid in which the macrophage count was within the same range as that for the normal fluids.

Spina bifida amniotic fluids: There were fluids from only two pregnancies in this group. They both had macrophage counts greater than the normals but less

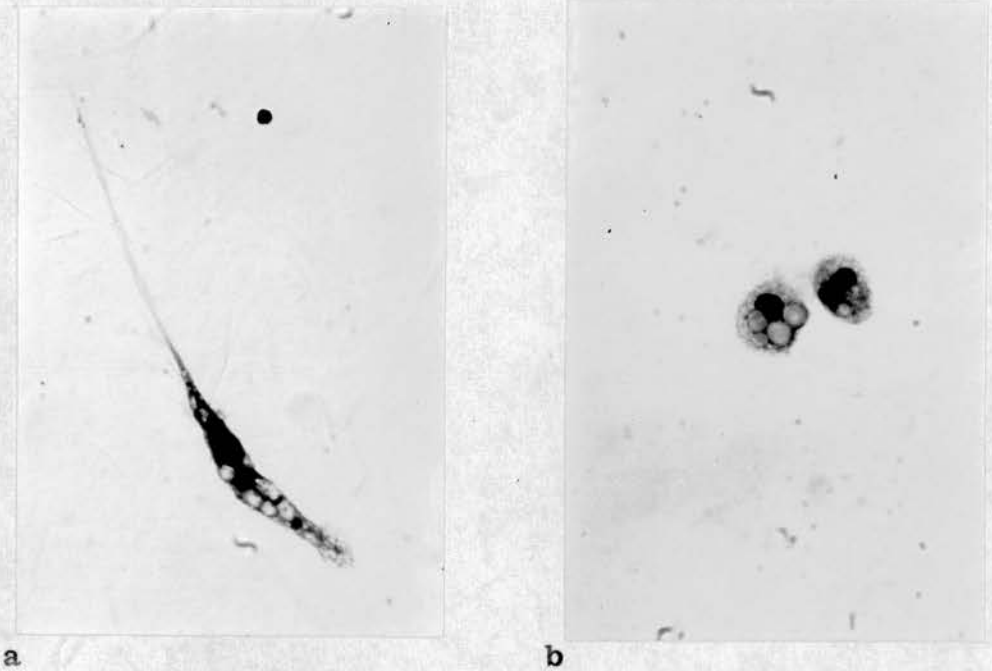
than the anencephalics. Their macrophage counts were in the same range as the higher values for the Rhesus iso-immunised fluids. The first such fluid (367) was collected primarily for chromosome studies, from a 26-year-old woman who had a child with Down's syndrome. The macrophage count was 168 and the α-fetoprotein was elevated (181 μg/ml). The pregnancy was terminated and resulted in a female fetus with spina bifida and meningomyelocoele. Amniotic fluid obtained at termination of pregnancy had a macrophage count of 189, in close agreement with the count from the first sample. The second pregnancy was to a 39-year-old woman who had amniocentesis performed for the antenatal diagnosis of maternal age associated chromosome anomalies. Amniocentesis at 13 weeks' gestation yielded fluid with a macrophage count of 82 and an α-fetoprotein of 143 μg/ml. The pregnancy was terminated and resulted in a female fetus with spina bifida and meningomyelocoele. Amniotic fluid obtained at termination was grossly bloodstained and had a macrophage count of 84 and an α-fetoprotein of 118 μg/ml.

Diabetic amniotic fluid: Only one such fluid was available for study and this had a normal macrophage count. The fluid was collected at 38 weeks' gestation for estimation of lecithin/sphingomyelin ratio from a woman who was a mild diabetic. The placenta showed no pathological changes (Dr. G.A. Machin).

Other amniotic fluids: Two samples of amniotic fluid

have been considered apart from all the others. These both had raised macrophage counts but normal α-fetoprotein levels. There was no evidence of feto-maternal incompatability or diabetes in either case. The reasons for the high macrophage count with the normal α-fetoprotein remain unknown. The pregnancies have been allowed to continue.

If the estimation of the macrophage count is to be usefully employed for the screening of amniotic fluid cultures then removal of a coverslip must be shown not to prejudice the success of the culture or to increase the time required to achieve a chromosome result. In only one case where a coverslip was removed for a macrophage count did the culture fail to produce either a chromosome result or a cell strain if this was required. This was a sample of fluid (376) grossly contaminated with old blood from a previously unsuccessful amniocentesis and with fresh blood; cellular proliferation in culture was not observed. The mean time to chromosome result for the first 25 normal cultures on which macrophage counts were performed was 13.0 days. The 20 cultures harvested in the minimum time prior to this, when macrophage counts were not being performed, required an average of 14.5 days for a chromosome result. These two figures are not strictly comparable since they were from cultures studied at different periods of time and there was a tendency for the time required for a cytogenetic result to fall (see chapters 2 and 4).



a b

Fig. 5.4. Two morphologically distinguishable types of macrophage showing erythrophagocytic activity (a) spindle shaped cell and (b) round cell. (Giemsa, x400).

However they do indicate that carrying out macrophage counts does not increase the time required for the chromosome results. Two grossly bloodstained samples yielded normal macrophage counts, hence this does not appear to interfere with the reliability of the count.

The nature of the macrophages in amniotic fluid.

All the data discussed above has related to the glass adherent cells in the amniotic fluid and to denote all of these as macrophages is probably incorrect. Morphologically there appears to be two types of cells which are capable of phagocytosis, a spindle-shaped cell which can have very long cytoplasmic processes (Fig. 5.4a) and a rounder type of cell which does not show cytoplasmic processes (Fig. 5.4b). These two types are probably the ends of a spectrum of cell morphology since numerous intermediate forms were also

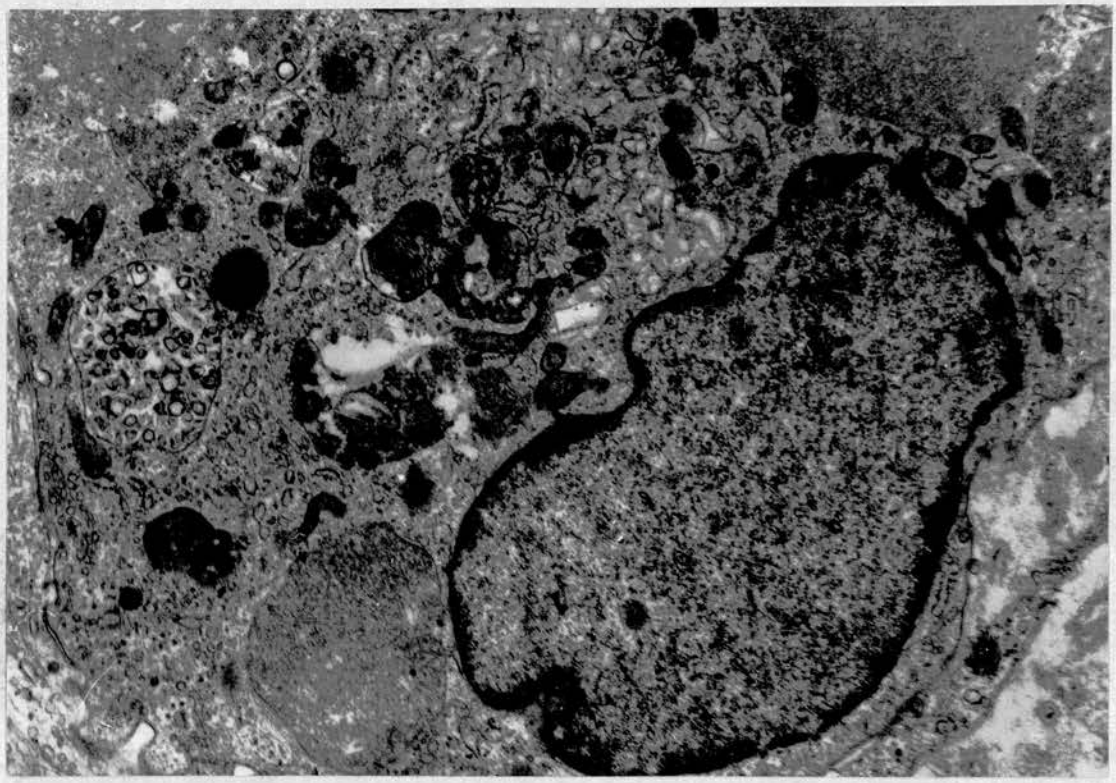


Fig. 5.5. Glass adherent cell from spina bifida amniotic fluid (369) after 48 hours in culture, showing features of a macrophage.
Note numerous phagosomes and myelin figure.
(Fixation: 2 parts 1% osmic acid and 1 part 2.5% glutaraldehyde in 0.067 M cacodylate buffer pH 7.4.
Stain: 1% uranyl acetate in 75% ethanol during dehydration and Reynold's lead citrate on section.
Embedded in Epon 812 resin. Electronmicrograph x10,000)

present. Initial studies showed that most of these cells were definitely of fetal origin (Sutherland et al., 1973b, appendix III). There is no way of determining the nature of all the glass adherent cells in amniotic fluid. Ultrastructural studies of some of these cells from the first case of spina bifida showed features which indicated that they were macrophages (Fig. 5.5) although some cells had ultrastructural features similar to those of the reticular cells (Fig. 5.6) described by Stuart and Davidson (1971).

Erythrophagocytosis studies on three Rhesus iso-

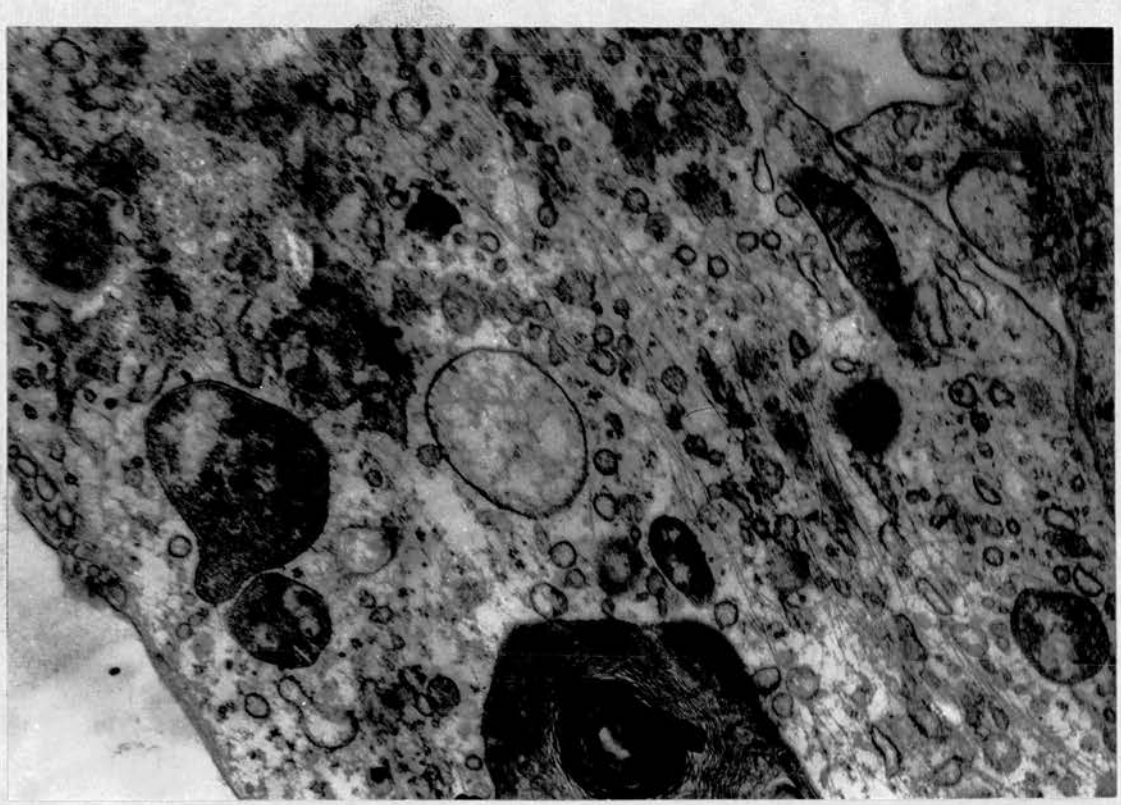


Fig. 5.6. Area of cytoplasm of glass adherent cell from spina bifida amniotic fluid (369) after 48 hours in culture, showing features of a reticular cell. Note numerous microfilaments, phagosomes and small vesicles.
(Preparation as for Fig. 5.5. Electronmicrograph x20,000)

immunised, two anencephalic and one spina bifida fluid showed that a proportion of the glass adherent cells were phagocytes. In the case of the spina bifida fluid erythrophagocytic activity was demonstrated after a total of 6 hours in culture (the last two hours in the presence of tagged red cells). Such studies are particularly difficult in the case of normal amniotic fluids because of the very low cell densities and the need to conserve most of the material for diagnostic cytogenetic studies. Because of the low cell densities it was difficult attempting to quantitate the erythrophagocytosis but the fluids from the cases

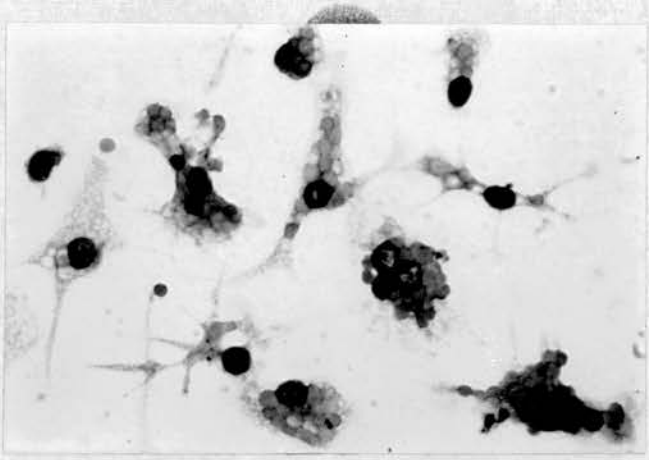


Fig. 5.7. Macrophages from hydrocephalic C.S.F. showing massive autoerythrophagocytic activity. (Giemsa, x400).

of CNS defects appeared to have about 60-80% of the glass adherent cells capable of phagocytosis. In the case of the Rhesus iso-immunised fluids only about 40-50% of the cells showed phagocytic activity.

In the anencephalic fluids in which the glass adherent cell density was high there were dividing cells present after 24-48 hours (ascertained by harvesting for cytogenetic studies after 6 hours exposure to colchicine). It was not possible to ascertain whether or not the dividing cells were macrophages. In those anencephalic fluids where the cell density was lower, and in some of the Rhesus iso-immunised fluids with high glass adherent cell density, metaphases were not seen after 6 hours exposure to colchicine. In one of the cases of spina bifida occasional metaphases were seen after exposure to colchicine overnight, but again it is unknown whether the dividing cells were macrophages.

Studies on CSF collected from cases of hydrocephalus at perinatal necropsy showed that macrophages were

abundant in this material (Fig. 5.7). There were some differences in the behaviour of these macrophages compared with those from amniotic fluid. Even when present at very high density CSF macrophages did not yield metaphases on exposure to colchicine. Auto-erythrophagocytosis was extensive in some of the CSF macrophage cultures but was rarely seen in amniotic fluid macrophage cultures.

DISCUSSION

This study of macrophages in amniotic fluid has shown that the macrophage count (i.e. the glass adherent cell count after 20 hours in culture) can be used as an indicator of gross fetal C.N.S. abnormality without prejudicing other diagnostic studies. Nelson et al. (1974) have confirmed the finding of increased macrophages in anencephalic amniotic fluid and used this to make an antenatal diagnosis of anencephaly. Unfortunately there are conditions which interfere and produce macrophage counts within the spina bifida range. Hence it is suggested that there are a number of possible instances in which macrophage counts might be increased:

1. Open fetal C.N.S. defects in which the C.S.F. is in free communication with the amniotic fluid. In such cases the α-fetoprotein level will also be increased. These cases present no problem for antenatal diagnosis.

2. Fetal lesions which result in an area of the fetus which is not covered with skin but with a membrane, such as exomphalos may produce raised macrophage levels in the presence of normal α-fetoprotein levels. There are two cases in the present series which could come into this group but until more experience is gained with this technique these cases present an antenatal diagnostic problem.

3. Rhesus iso-immunisation (and presumably any other feto-maternal incompatability) can result in a

moderate increase in the macrophage count. This increase in amniotic fluid macrophages is associated with the increase in placental macrophages (Hofbauer cells) reported in this condition (Fox, 1967). Both these findings might be due to the increase in fetal extramedullary haemopoesis which can occur in this condition (Morison, 1963). If this is the case then increased amniotic fluid macrophage counts could be expected where there is maternal diabetes mellitus since both increased extramedullary haemopoesis (Morison, 1963) and increased Hofbauer cells (Fox, 1967) are known to occur in this condition. Only one diabetic amniotic fluid was available for study and its normal macrophage count does not impede this argument since it was from a mild diabetic and there was no placental pathology.

4. If the argument regarding an increase in extramedullary haemopoesis is valid then an increase in amniotic fluid macrophages would be expected in any other condition in which this was a feature, such as the chondro-dystrophies (Potter, 1961) and some intra-uterine infections (Bain et al., 1956).

Hence the estimation of the amniotic fluid macrophage count may be a useful indicator of fetal diseases other than those involving gross lesions. Unfortunately interference with this count as a diagnostic test by conditions which are generally recognised as compatible with normal survival may be a

problem. However a raised macrophage count can suggest that all is not well and indicate that further investigation is required. Sufficient experience has not yet been gained with cases in which there is a raised macrophage count in the presence of normal α-fetoprotein levels and the absence of Rhesus incompatability or maternal diabetes. The numbers involved in the present study are too small to indicate how often this situation may arise. Even on the present evidence it is considered that a raised macrophage count without explanation could be grounds for therapeutic abortion if it is accepted that termination of a possibly normal pregnancy is preferable to allowing the birth of an abnormal infant.

The origin of the macrophages in the amniotic fluid remains speculative. There are a number of possibilities in both the normal and abnormal cases. It is worth considering the absolute number of macrophages present. In an amniotic fluid with a macrophage count of 20 (about the middle of the normal range) there would be 125 macrophages/ml. At, for example, 20 weeks' gestation the volume of amniotic fluid is about 450 ml. (Emery, 1970), therefore the total number of macrophages would be about 60,000. This is not a great number of cells and with a motile cell such as the macrophage migration via the lungs, urinary tract and even the placenta itself could account for these. Even in the Rhesus iso-immunised cases, where there is no obvious point of entry of the macrophages into the amniotic fluid

the numbers encountered were only about 10 times normal, i.e. 600,000 cells. Hence if the increase in haemopoesis augmented the number of circulating macrophages then again simple migration could be responsible for the increased macrophage count.

In the cases with open C.N.S. lesions the C.S.F. is a possible source of the macrophages since it can contain these cells. In anencephaly, and to a lesser extent in spina bifida, there is a large area of highly vascularised tissue exposed and there can be little to impede migration of circulating macrophages into the amniotic fluid.

The nature of the glass adherent cells remains unclear. There can be little doubt that a proportion of these are macrophages in view of the rapidity with which they become attached to glass, their erythrophagocytic capacity and their ultrastructure. Morphologically they are similar to the human peritoneal macrophages described by Stuart (1967). There is controversy about the ability of mature macrophages to divide in vitro (Jacoby, 1965) although a recent report (Golde et al., 1974) claimed that mature pulmonary macrophages could proliferate in culture. Stuart (1967) described another cell type present in macrophage cultures, a serosal or reticular cell which was capable of undergoing division. Some of the cells in the present study had an ultrastructure similar to that of these reticular cells (Stuart and Davidson, 1971; Davidson, personal communication). Further

characterisation of the glass adherent cell population in amniotic fluid is required and would probably be best pursued using histochemical studies and by an examination of the cell surface receptor sites.

Hence it has been shown that the quantitation of glass adherent cells in amniotic fluid, whether all of these cells are macrophages or not, can be a useful diagnostic aid. The full potential of this finding is yet to be realised. In laboratories without facilities for α-fetoprotein determination but with tissue culture capabilities this can be, and has been (Nelson et al., 1974) used for the antenatal diagnosis of gross C.N.S. abnormalities. When there are facilities for α-fetoprotein studies the macrophage count can provide an independent parameter on which to base the antenatal diagnoses of gross C.N.S. abnormalities. Raised macrophage counts due to other causes are a problem at present and will remain so until much more information about these cases is available. It is possible that raised macrophage counts may be a non-specific indicator that all is not well with the fetus and suggest that further investigation is required.

SUMMARY

The glass adherent cell population of amniotic fluid has been quantitated. Many of the glass adherent cells have been shown to be macrophages and the glass adherent cell count has been referred to as the macrophage count. Greatly increased macrophage counts have been shown to occur in anencephaly. A lesser but definitely increased count occurs in spina bifida and can occur in Rhesus iso-immunisation. Two cases have been encountered with increased macrophage counts of unknown etiology and the pregnancies have been allowed to continue. It has been shown that amniotic fluid cultures can be screened by macrophage counts without prejudicing their success. The potentialities of this parameter as a non-specific diagnostic test of extra-medullary fetal haemopoesis as well as a specific test for fetal C.N.S. abnormalities is discussed.

Chapter 6

Lysosomal Enzymes in Cultured Amniotic Fluid Cell Strains

BACKGROUND

Nadler (1968b) and Fujimoto et al. (1968) showed that some inborn errors of fetal metabolism could be identified in cultured amniotic fluid cells. Before this knowledge can be applied to antenatal diagnosis of genetic diseases it is essential that the normal characteristics of any enzyme that may have to be measured for such diagnosis are known. Indeed Nadler (1968a) wrote "Knowledge of normal patterns of enzyme distribution in cultured amniotic fluid cells is mandatory if we are to utilize this material in a responsible manner." It is not sufficient to extrapolate data gained from the study of cultured skin fibroblast-like cells to cultured amniotic fluid cells since the biochemistry of these two types of cell strain are not identical. Even within amniotic fluid cell strains the biochemistry of the various cell types is different (Gerbie et al., 1972). Melancon et al. (1971) showed that "epithelial cells" from amniotic fluid cell strains had high levels of histidase whereas "fibroblasts derived from the same original culture" had no detectable activity of this enzyme. Conversely cystathionine synthetase is active in cultured fibroblast-like cells but not in epithelioid cells from amniotic fluid (Gerbie et al., 1972). This latter finding is somewhat at variance with that of Uhlendorf and Mudd (1968) who found that this enzyme has a much higher activity in cultured amniotic fluid cells than in cultured skin fibroblast-like cells. Brock (1973) lists 12 enzymes for which relative activities in these

Table 6.1. Lysosomal enzymes chosen for study in cultured amniotic fluid cells and the inborn errors of metabolism with which they are associated.

Lysosomal enzyme	Disease*
N-Acetyl-ß-D-glucosaminidase	Tay-Sachs complex
Acid Phosphatase	Lysosomal acid phosphatase deficiency
ß-galactosidase	Generalised gangliosidosis
ß-glucuronidase	ß-glucuronidase deficiency
ß-glucosidase	Gaucher's disease
α-galactosidase	Fabry's disease
α-arabinosidase	Not known yet
α-glucosidase	Pompe's disease
α-mannosidase	Mannosidosis
Arylsulphatase A	Metachromatic leukodystrophy

* Brock (1972) has tabulated references to the identification of these diseases except for ß-glucuronidase deficiency which was described by Sly et al. (1973).

two types of cell strain have been reported.

In view of this need to know more about the characteristics of enzymes in cultured amniotic fluid cells a group of lysosomal enzymes was chosen for study. All except one of these is involved in an inborn error of metabolism (Table 6.1). These particular enzymes were chosen because there are simple and reliable assay methods available for them and because they are relevant to both post-natal and antenatal diagnosis.

The first studies on lysosomal enzymes in cultured amniotic fluid cell strains were by Nadler (1968b).

Although his paper contained no assay values for acid phosphatase, α-glucosidase, β-glucuronidase and β-galactosidase, he claimed that the levels of these enzymes in cultured amniotic fluid cells did not vary with gestation over the period from 10 to 36 weeks. He also claimed that the activities of these enzymes in cultured amniotic fluid cells were not different from those found in (presumably) cultured fibroblast-like cells from newborns and adults.

There has been no study of the levels of activity of lysosomal enzymes in normal amniotic fluid cell strains. The only such data in the literature are in case reports of antenatal diagnoses which include a few "control" cell strains in which the relevant enzyme was assayed or from studies of individual enzymes where the level of the particular enzyme has been measured in a number of tissues including cultured amniotic fluid cells. Brock (1973) has listed 18 enzymes (mostly lysosomal) for which assay values in cultured amniotic fluid cells have been quoted.

Since the enzymology of cultured amniotic fluid cells cannot be assumed to be the same as that of cultured fibroblast-like cells it is necessary for the specific activities of the enzymes in both of these types of cell strain to be measured and their relationship known. The main reasons for this are that in many antenatal diagnoses the only suitable control material available for the enzyme assay is cultured fibroblast-like cells. In addition, confirmation of an antenatal diagnosis may have to be made using such material. This

problem has been discussed at some length by Kaback and Leonard (1970) who compared activities of ß-galactosidase, N-acetyl-ß-D-glucosaminidase and arylsulphatase A in cultured amniotic fluid cell strains with that in maternal and fetal fibroblast-like cell strains. For each enzyme they found that the activity in fetal fibroblast-like cell strains was significantly less than that in amniotic fluid cell strains. They also found that ß-galactosidase activity was higher in cultured amniotic fluid cells than in maternal fibroblast-like cell strains and, conversely, that arylsulphatase A activity was lower in cultured amniotic fluid cells. The results of Kaback and Leonard (1970) are not supported by Leroy et al. (1973) who found

Table 6.2. Reported antenatal diagnoses of inborn errors of metabolism of the lysosomal enzymes included in this study.

Disease	First antenatal diagnosis*
Tay Sachs	Schneck et al. (1970); O'Brien et al. (1971)
Sandhoff's	Desnick et al. (1973)
Lysosomal acid phosphatase deficiency	Nadler and Egan (1970)
Generalised gangliosidosis	Lowden et al. (1973)
Gaucher's	Epstein et al. (1972)
Fabry's	Brady et al. (1971)
Pompe's	Nadler and Messina (1969)
Metachromatic Leuko-dystrophy	Nadler and Gerbie (1970); Leroy et al. (1973)

* Where two references are given either the first gives inadequate documentation or the second is a key reference related to the particular disease.

the same level of activity in amniotic fluid cell strains and cultured skin fibroblast-like cells.

Another problem in the enzymology of cultured amniotic fluid cells is that of cell type (see Sutherland et al., 1974a, appendix III). Kaback and Leonard (1972) assayed ß-galactosidase, arylsulphatase A and N-acetyl-ß-D-glucosaminidase in cells of epithelioid and fibroblast-like morphologies and found no differences between the two groups. Gerbie et al. (1972) did the same for a number of enzymes and claimed that there were no differences in the lysosomal enzyme activities of these cell types. Examination of the data of Gerbie et al. (1972) suggests that their claim is not true for all the enzymes. For acid phosphatase there is no overlap of the quoted ranges of activity in the two cell types.

Antenatal diagnoses of 9 of the inborn errors in which the enzymes chosen for study are involved have been reported (Table 6.2). In spite of this there has been no systematic study of factors associated with tissue culture, such as passage number, time after subculture, growth rate of the cells, type of culture medium, amount of serum supplement to the culture medium or pH of the medium which could affect the enzymology of cultured amniotic fluid cells.

MATERIALS AND METHODS

The materials used in this study were the cell strains derived from amniotic fluid cell cultures as described in chapter 3. The methods of cell homogenisation and enzyme assay are included in the section of this work which has been published (see appendix III). Two methods of cell homogenisation were used. The first was used for the majority of the studies and is outlined in Butterworth et al. (1973a, appendix III). The second was used for the later phases of the study and is outlined in Sutherland et al. (1974b, appendix III).

RESULTS

Most of the results of this study have been published. The levels of the lysosomal enzymes in normal cultured amniotic fluid cell strains at the third passage of culture are given in Butterworth et al. (1973a , appendix III). Studies of the variation of the levels of these enzymes with time in culture are reported in a series of three papers, Butterworth et al. (1973b), Sutherland et al. (1974c) and Butterworth et al. (1974a), all in appendix III. The effects of serum concentration in the culture medium, the type of culture medium used and the pH of the culture medium on the levels of the lysosomal enzymes in cultured amniotic fluid cells are presented in Butterworth et al. (1974b , appendix III). A study of the variations in enzyme levels in thirteen cell strains derived from one sample of amniotic fluid using three culture methods (see chapter 2) are reported in Sutherland et al. (1974b , appendix III). The last two studies were carried out using cell homogenates prepared by the second method of cell homogenisation (Sutherland et al., 1974b).

The only part of this study which has not been published is a comparison of the effects of the two homogenisation methods on the activities of the lysosomal enzymes and this is shown in table 6.3.

The method of homogenisation did not affect the specific activities of acid phosphatase, α-galactosidase or α-mannosidase. The activities of three enzymes,

Table 6.3. Comparison of the two methods of cell homogenisation used on the specific activities* of the lysosomal enzymes studied.

Enzyme	Method I (Butterworth et al. 1973a) n = 38-41	Method II (Sutherland et al. 1974b) n = 14
N-acetyl-ß-D-glucosaminidase	52.1 ± 16.7[a] 28.7 - 91.9[b]	37.2 ± 16.1*** 23.0 - 88.9
Acid Phosphatase	7.73 ± 2.82 4.34 - 12.69	7.43 ± 3.25 4.35 - 15.75
ß-galactosidase	4.59 ± 1.97 2.37 - 10.69	6.02 ± 2.15**** 2.75 - 10.96
ß-glucuronidase	1.30 ± 0.52 0.62 - 2.02	0.74 ± 0.26** 0.31 - 1.40
ß-glucosidase	0.072 ± 0.030 0.03 - 0.17	0.17 ± 0.079** 0.06 - 0.26
α-galactosidase	0.42 ± 0.15 0.22 - 0.78	0.41 ± 0.12 0.27 - 0.57
α-arabinosidase	0.53 ± 0.25 0.22 - 1.30	0.17 ± 0.077** 0.10 - 0.34
α-glucosidase	0.38 ± 0.18 0.17 - 0.97	0.66 ± 0.28** 0.36 - 1.49
α-mannosidase	0.65 ± 0.30 0.28 - 1.27	0.85 ± 0.41 0.36 - 1.78

* nmole methylumbelliferone/min./mg. protein

** $P < 0.001$ *** $P < 0.01$ **** $P < 0.05$

a mean ± standard variation b range

N-acetyl-ß-D-glucosaminidase, ß-glucuronidase and α-arabinosidase were lower when the second method of extraction was used, whereas the activities of ß-galactosidase, ß-glucosidase and α-glucosidase were lower when the first method was employed.

During the course of this study three women at risk for having children with inborn errors of metabolism

were studied.

Case 1. Mrs. S.M. had one normal child and one child which died in the neonatal period and was diagnosed as suffering from maple syrup-urine-disease. She became pregnant in October 1971, amniocentesis was performed at 15 weeks' gestation and amniotic fluid (42) cell culture established. The culture grew slowly and then degenerated. Because of failure to reach any decision about the status of the fetus the pregnancy was terminated and resulted in a macerated male fetus. No decision was reached as to whether this fetus was normal. Mrs. S.M. again became pregnant in September 1972 and amniocentesis was performed at 15 weeks' gestation. The amniotic fluid (205) culture grew well and after 4 weeks assays for leucine decarboxylase (Dr. R.A. Harkness) indicated that normal amounts of this enzyme were present. The pregnancy continued and at term a female infant was delivered. Serum and urine amino acid analyses indicated that this infant had maple syrup urine disease. Enzyme studies on cord blood and venous blood showed normal levels of leucine decarboxylase activity. The infant is surviving with dietary treatment for its inborn error of metabolism.

Case 2. Mrs. McD. was at risk for producing a child with Sandhoff's disease, full family studies having been carried out in this department (Bain et al. 1973). She became pregnant in April 1973 and amniocentesis was performed at 14 weeks' gestation. The amniotic fluid

(284) was grossly bloodstained and after one week in culture there was no evidence of cellular proliferation. A second amniocentesis was performed 6 days after the first and again the amniotic fluid (286) was bloodstained. The cells from this sample grew slowly in tissue culture and five weeks after amniocentesis there was one small baby bottle of cells available for enzyme assay. Assay of N-acetyl-ß-D-glucosaminidase indicated that this enzyme was present and the pregnancy was allowed to continue. Assay values for this enzyme in both samples of amniotic fluid and the cultured cells are shown in table 6.4. The infant was subsequently shown to have normal levels of N-acetyl-ß-D-glucosaminidase in its cord blood leucocytes.

Table 6.4. Results of enzyme studies on which the antenatal diagnosis in case 2 were based.

Material		Specific activity of N-acetyl-ß-D-glucosaminidase
Amniotic fluid	284	3.80[a]
	286	3.06[a]
Cultured cells	286	31.4 [b]
	1	43.7 [b]
	2	51.5 [b]
	3	57.8 [b]
	Sib	1.00[b]

a nmole methylumbelliferone/min./ml. of fluid.
b nmole methylumbelliferone/min./mg. protein.
1, 2 and 3 are normal amniotic fluid cells cultured at the same time as 286.
"Sib" is a fibroblast-like cell strain from a previously affected sibling of the fetus.

Case 3. Mrs. L.C. had two children with a neurodegenerative disorder which had been diagnosed as metachromatic leukodystrophy on the basis of low levels of the enzyme arylsulphatase A. An attempt at antenatal diagnosis in another hospital had been inconclusive. Amniotic fluid (291) from the present pregnancy was received at 23 weeks' gestation. The culture grew well and enzyme assays had been completed within 4 weeks. Arylsulphatase A was present and the pregnancy was allowed to continue, a healthy female infant was delivered. The results of the enzyme assays on which this diagnosis was based are shown in table 6.5. The infant had normal levels of arylsulphatase A in its cord blood leucocytes.

Table 6.5. Results of enzyme studies on which the antenatal diagnosis in case 3 was based.

Material		Arylsulphatase A specific activity[a]
Cultured cells 291	I	0.127
	II	0.210
Normal control		0.116
Affected control		0.001

[a] nmole nitrocatechol/min./mg. protein.

Two separate cultures (I and II) of amniotic fluid cells were assayed, one normal control amniotic fluid grown under the same conditions and fibroblast-like cells from an unrelated affected control were assayed at the same time.

DISCUSSION

The sections of this study which have been published have all been discussed in the relevant papers in appendix III. This work showed that there were very marked fluctuations in the levels of the enzymes studied at different stages of culture. In spite of investigation of some of the variables which might have been responsible for this fluctuation the reasons for it remain unknown. The relevance of these findings to the antenatal diagnosis of inborn errors of metabolism by enzyme assay of cultured amniotic fluid cells is that this fluctuation could be a source of error. In the present study no cell strain at any time had such a low level of enzyme activity that it could have been suspected of having an enzyme deficiency. The chances of any of the cell strains studied being heterozygous for a lysosomal enzyme deficiency are small. In pregnancies where the fetus is at risk for an inborn error of metabolism half the amniotic fluid cell strains could be expected to be heterozygous. If the enzyme levels in these cell strains, which would be expected to be about half that of normal cell strains, fluctuate to the same extent as they do in these latter cell strains then such fluctuations might lead to the diagnosis of an enzyme deficiency if only an isolated cell culture was assayed. No antenatal diagnosis of an inborn error of metabolism should be established by the assay of the cells from a single culture bottle of amniotic fluid cells. The particular cell strain involved should be

assayed at least twice at different stages of culture before an enzyme is considered to be absent.

The preparation of cultured cells for enzyme assay is a problem which almost all workers solve differently. Even different reports from the one laboratory use different methods of cell homogenisation even when the same group of enzymes are being studied. Gerbie et al. (1972) removed cells from the culture vessel with trypsin, washed them in isotonic saline and then suspended them in water. On the other hand Ryan et al. (1972), essentially the same group of workers, suspended their cells in 0.25M sucrose, lysed them by 5 cycles of freeze-thawing then centrifuged at 600g for 10 min to remove cell debris. It is of some concern that Gerbie et al. (1972) obtained activities of β-glucosidase 50 times higher, and arylsulphatase about 5 times higher in cultured amniotic fluid cells than Ryan et al. (1972) found in fibroblast-like cell strains when using the same conditions of enzyme assay. Other variations have included sonication in phosphate buffered saline followed by centrifugation at 200g (Kaback and Howell, 1970), freezing in isotonic saline followed by sonication (Milunsky et al., 1972a), sonicating in water (Kanfer and Spielvogel, 1972) and suspension in water followed by mechanical homogenisation (Den Tandt and Schaberg, 1973). Beutler et al. (1971) used a saline suspension of intact fibroblasts for enzyme assay and claimed that freeze/thawing had no effect on enzyme levels.

Ryan et al. (1972) reported that excessive

centrifugation of homogenates removed ß-galactosidase activity. This report prompted the change in homogenisation used in the present study. The findings of Ryan et al. (1972) were confirmed as far as ß-glucosidase activity was concerned in that the enzyme activity in homogenates centrifuged at the lower speed were significantly higher than in those centrifuged at 25,000g.

There are a number of possible explanations for the effects of the homogenisation methods used in the present study. When enzyme levels remain constant the distribution of the enzyme must be the same in the particulate fraction and the soluble fraction of the homogenate. When enzyme levels are higher in the homogenate produced by the second method this may be because protein removed by the high speed spin does not contain enzyme, hence the specific activity, expressed on a protein basis, will be higher. The reasons for enzyme levels being lower in the homogenate produced by the second method are that the enzyme is being concentrated in the high speed sediment, this could be for reasons of solubility or because the enzyme has to be membrane bound to function normally (Ho et al., 1973). The possibility that the NaCl used in the first method may increase the solubility of some enzymes or act to stabilise them cannot be discounted. In addition, sonication could affect enzyme activity. Some of the enzymes in the group studied are considered to be constituent parts of the lysosomal membrane and others

to be bound to it (Tappel, 1969). For example ß-glucosidase remains firmly bound to the membrane of rat liver lysosomes when these are ruptured (Beck and Tappel, 1968). This whole problem of cell homogenisation requires further study and standardisation if results from different laboratories are to be comparable.

The three pregnancies monitored for possible inborn errors of fetal metabolism illustrate several important points which should be noted. The first case involving "maple-syrup-urine disease" should never have been attempted. The basic requirement that the previously affected individual in the family should have been diagnosed at the enzyme level was not met. In this first case it would appear that because this basic requirement was not fulfilled the antenatal diagnosis had no chance of being correct other than the fortuitous one of being right for the wrong reason, since the infant appears to have normal levels of the enzyme which was thought to be absent. Others have been more successful in the antenatal diagnosis of maple-syrup-urine disease. Nadler and Gerbie (1970) monitored two pregnancies at risk for this disease and predicted that the fetuses would be normal. Milunsky (1973) records that seven pregnancies had been monitored for this condition, one was terminated after being diagnosed as affected and the remaining six were unaffected. The only adequately documented instance of the antenatal diagnosis of this disease is the case reported by Wendl et al. (1973).

The second case is probably as close to an ideal

situation as is likely to be encountered in this type of work. The family had been studied previously in this department (Bain et al., 1972) and cultured skin fibroblasts from one of the previously affected members were available for use as control material for the enzyme assay. Desnick et al. (1973) have documented the antenatal diagnosis of Sandhoff's disease.

The third case has a number of unsatisfactory features. The amniotic fluid was received in mid-pregnancy after another laboratory had failed in an antenatal diagnosis attempt. The family had not been studied enzymatically in this department and the residual enzyme activity in the affected members of the family was unknown. Metachromatic leukodystrophy has been subjected to antenatal diagnosis on a number of occasions; Milunsky (1973) records 6 pregnancies which have been monitored for this disease.

SUMMARY

The normal range of activity of 9 lysosomal enzymes in cultured amniotic fluid cells was established. Marked variations in the levels of the enzymes studied were found to occur throughout the period of culture of individual cell strains. The serum concentration of the culture medium, the type of culture medium (Ham's F10, Eagle's M.E.M.) and the pH of the culture medium did not affect the levels of the enzymes with the exception of a small but significant increase in the activity of ß-galactosidase when grown in medium with a serum concentration of 30% compared with 15%. The levels of the enzymes in 13 cell strains, at the third passage of culture, derived from a single sample of amniotic fluid showed less variation than did the enzymes in cell strains derived from different samples of fluid. Nevertheless some enzymes in these 13 cell strains showed 2 to 3 fold variations in activity. Three pregnancies in which the fetus was at risk for an inborn error of metabolism were monitored and a mistake in antenatal diagnosis is recorded.

Chapter 7

Urine Cell Culture

INTRODUCTION

The specific site of origin of the cells cultured from amniotic fluid is unknown. The heterogenous nature of amniotic fluid cultures suggests that more than one type of cell is proliferating. Cytological studies on amniotic fluid have indicated that the cells present come from various sources. Exfoliated cells from the amnion, fetal skin, respiratory and urogenital tracts have all been implicated (see Sutherland and Bain, 1972, appendix III for references). Even without cytological evidence it is difficult to imagine other possible sources of these cells. However, Uhlendorf (1970) has suggested that some of the cells may have originated from the chorionic villi.

The only experimental approach to this problem was made by Jacobson and Barter (1967). They initiated cultures from amniotic, allantoic and tracheal fluids and urine from sheep fetuses; only one of the urines produced a successful culture. They also initiated cultures from bladder aspirates of four human fetuses and chorionic and yolk sac fluids from three human fetuses; none of these cultures were successful.

Early in this work it was found that cells from the urine of newborn children could be used to initiate cultures (Sutherland and Bain, 1972, appendix III). These cultures could be readily used for cytogenetic studies (Sutherland et al. 1973a, appendix III). Hence it is feasible that some, although not necessarily all, of the cells from amniotic fluid which proliferate in culture

are derived from the fetal urinary tract.

A comparison of cultured urine cells has been made with cultured amniotic fluid cells at the morphological, cultural and biochemical levels. Cultured urine cells may be a more suitable model than cultured skin fibroblasts for biochemical studies which may have to be extrapolated for use in antenatal diagnosis of inborn errors of metabolism.

MATERIAL AND METHODS

Urines were collected into standard sterile urine collecting bags by the nursing staff. Occasional samples were collected by suprapubic aspiration, either when urine cell culture could be justified clinically or for bacteriological purposes when the excess was available for cell culture. Urine was collected from perinatal post-mortems by aspiration from the bladder after the abdomen had been opened.

The methods used for all phases of the work on urine cell culture were the same as those used for comparable studies on amniotic fluid cells.

RESULTS

The results of the studies on urine cell culture will be presented in the same sequence as the studies on amniotic fluid cell culture. Since the success rate in culturing urine cells was low the first section of these results will relate to possible factors affecting the success of urine cell culture. Microbial contamination of urine samples was a major problem in this work; about 25% of urines collected into urine collecting bags from newborn infants showed contamination in the first few days of culture. These samples will not be considered further. Success in culture is defined as the appearance of

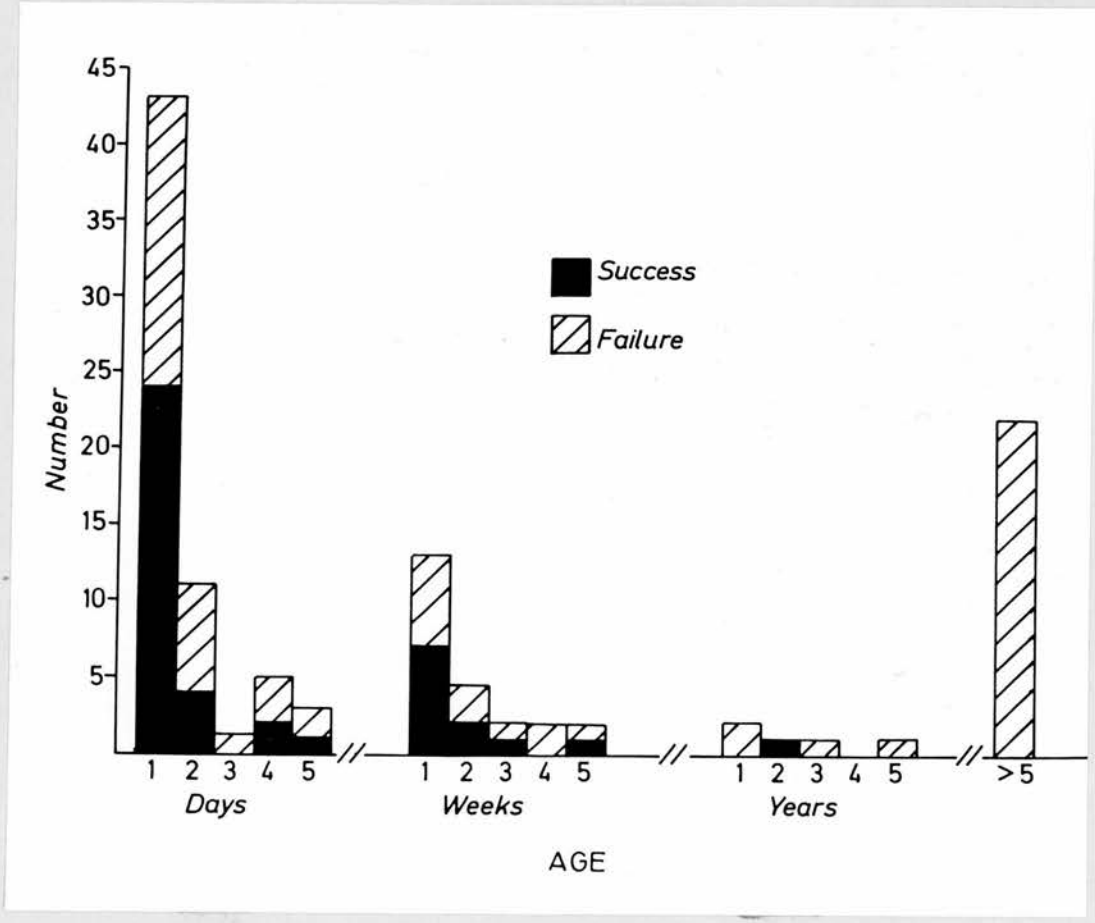


Fig. 7.1. Histogram showing the relative success rates of urine cell culture for individuals of varying age.

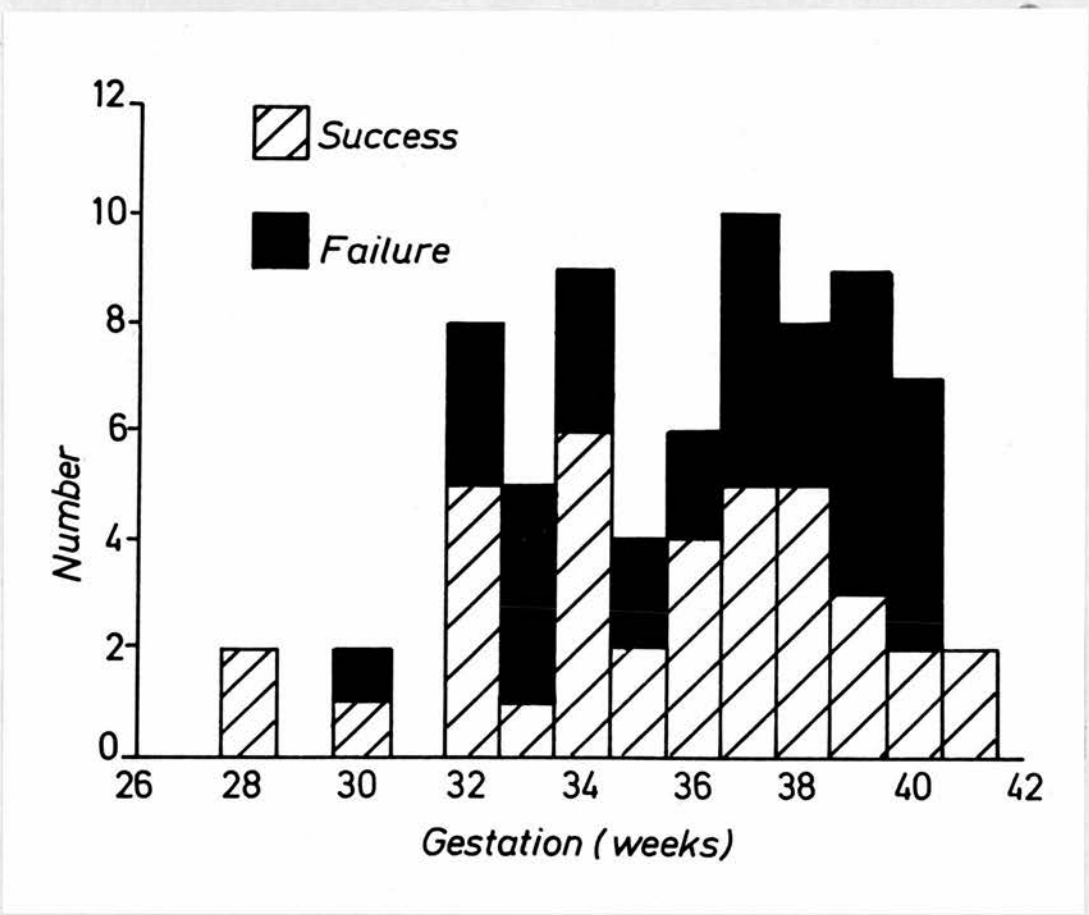


Fig. 7.2. Histogram showing the relationship between gestational age and success in urine cell culture for fetuses and infants aged one week or less.

proliferating cells in the Petri dish within one month and data pertaining to those urines successfully cultured are in table II, appendix II.

The relationship between the age of the individual from whom the urine was collected and success in culture is shown in Fig. 7.1. This figure does not include urines collected from stillborn fetuses at postmortem. The oldest child from whom urine cells were successfully cultured was 2 years old, the "youngest" was a fetus of 18 weeks' gestation. During the first few weeks of life, success in culture appeared to be unrelated to age, however for older children the chance of success in

culture was very low.

The relationship between the gestation of stillborn fetuses and of the infants aged one week or less to success in culture is shown in Fig. 7.2. One successful culture from the bladder aspirate of a fetus of 18 weeks' gestation has been omitted from this figure. The success of urine culture from fetuses and neonates does not appear to be related to their gestational age.

The relationship between the cell count of a group of urines and success in culture is shown in Fig. 7.3. As in the case of amniotic fluids, urines with very low cell counts were allocated a value of 1000 cells/ml. The cell counts appear to have little effect on the success of the urine culture. Urines with 1000 or less cells/ml. both succeeded and failed in culture as did urines with more than 100,000 cells/ml.

If all urines initially contain cells which have the potential to proliferate in tissue culture, then the nature of the urine itself could be such as to maintain or destroy this potential. Two properties of the urine were investigated in this regard, osmolarity and acidity.

The relationship between osmolarity and success in culture is shown in Fig. 7.4. Most of the successfully cultured urines had osmolarities on the hypotonic side of physiological normal (approximately 300 mosm./Kg.). The unsuccessful samples were also mainly on the hypotonic side of normal but some were more hypertonic. These low values for urine osmolarity are due to most of the samples being from infants in the first day or so of life.

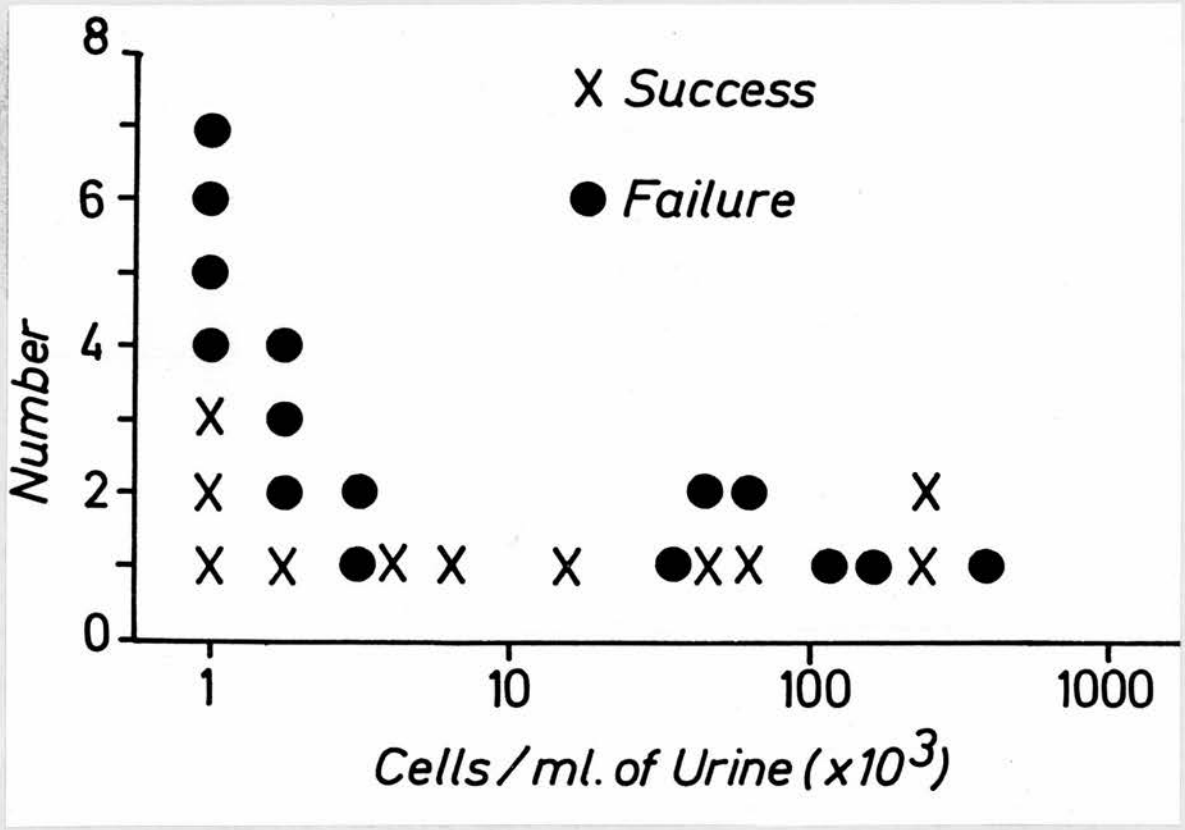


Fig. 7.3. Graphical display of the relationship between urine cell count and success of urine cell culture.

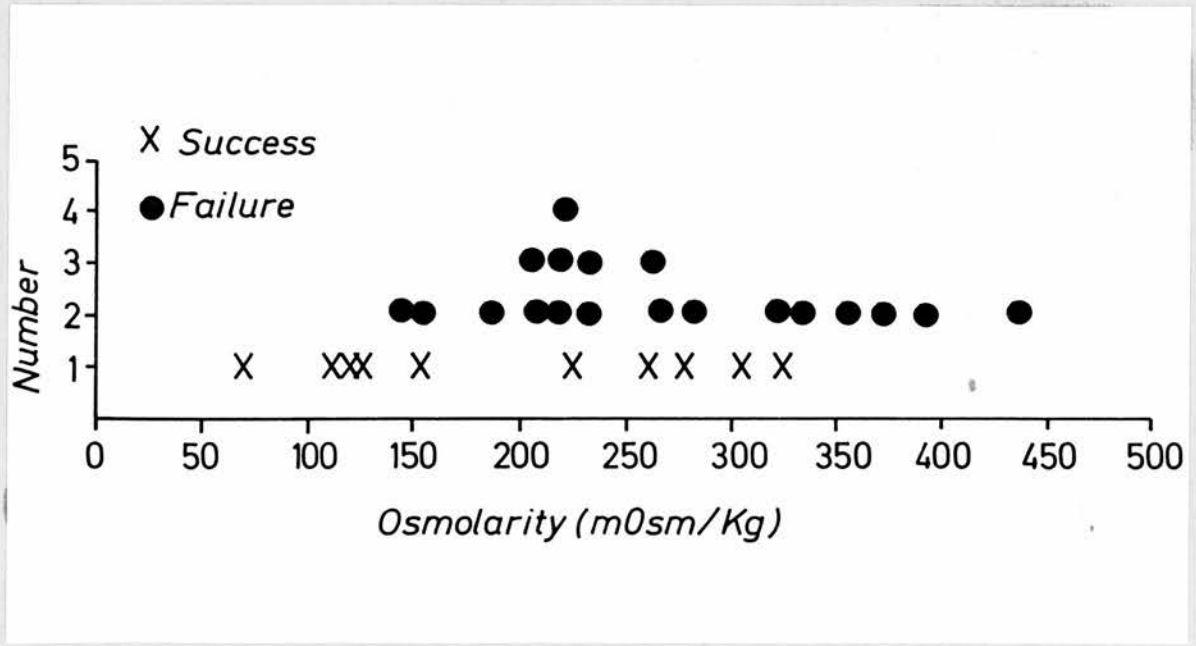


Fig. 7.4. Graphical display of the relationship between urine osmolarity and success of urine cell culture.

Table 7.1. Relationship between degree of acidity and success of urine cultures from infants less than one month old.

	Degree of acidity			
	0	+	++	+++
Success	8	1	2	-
Failure	10	2	3	7

The degree of acidity was rated by the colour change in the medium when it was mixed with the urine cells. From table 7.1 it can be seen that all urines which were rated as very acidic failed to grow, however those rated as not acidic were not necessarily successful in culture. The only tentative conclusion is that while hypertonic, acidic urines are unlikely to be successful in culture these two factors are not useful predictors of the outcome of culture when the urine is more neutral and isotonic.

There were 46 urines which were successful in culture

Table 7.2. Data on the origin of the 46 urines which were successfully cultured.

Means of collection*	Number	Age Range*	Number $\leq$ 7 days old	Gestational age range (weeks) for those $\leq$ 7 days old
(i)	16	1-23d	15	32 - 40
(ii)	24	SB-35d	22	18 - 40
(iii)	6	1d-2y	2	35 - 38
Total	46	SB-2y	39	18 - 40

* See Table II, appendix II.

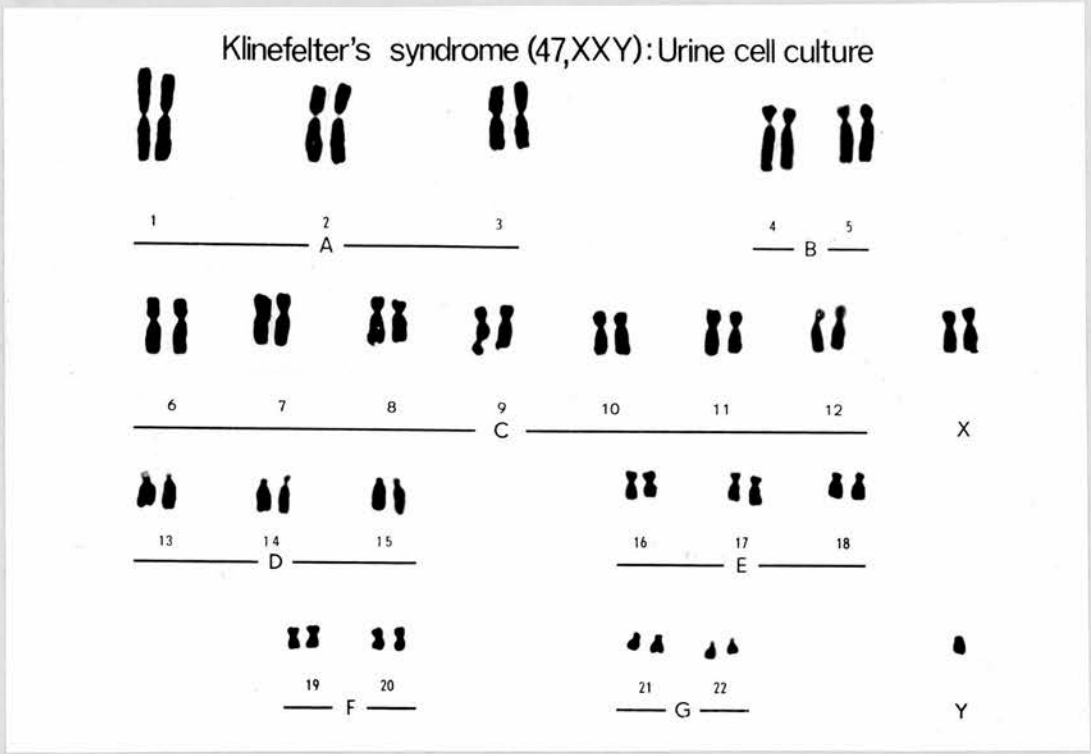


Fig. 7.5. Karyotype from primary urine cell culture from a male infant which had been dead for 5 days prior to postmortem. Note 47,XXY karyotype.

and all further results pertain to these. Some data on the origin of these 46 urine samples are given in table II, appendix II and summarised in table 7.2.

Cytogenetic studies were carried out on 29 of the successfully cultured urines. Three of these urines had abnormal karyotypes, one (93) was 47,XXY (Fig. 7.5) and two samples (116 and 130) were from an infant with Edward's syndrome and karyotype 47,XX,+18/48,XX,+18,+fra (Fig. 7.6). These two infants had been diagnosed as chromosomally abnormal before urine cell culture was attempted. The use of urine cell culture for cytogenetic studies has been published (Sutherland et al., 1973a, appendix III).

Since cytogenetic results from urine cell culture

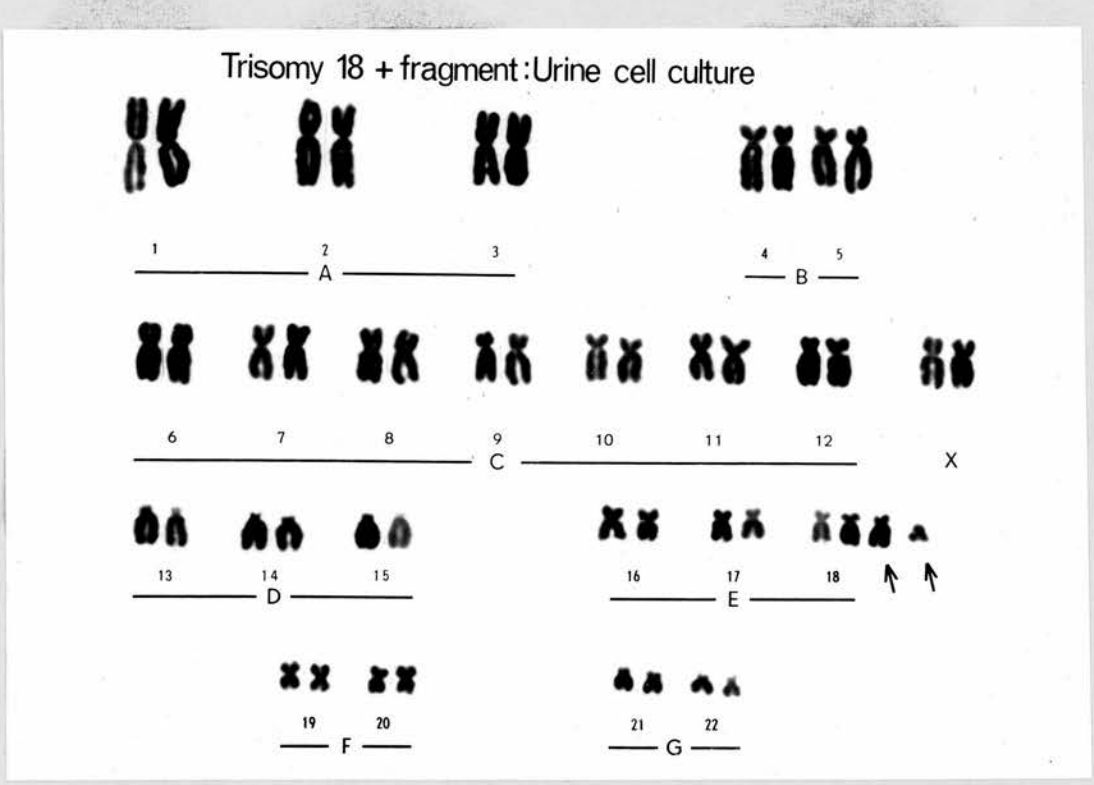


Fig. 7.6. Karyotype from the third passage of a cell strain derived from urine collected by suprapubic aspiration from an infant with Edward's syndrome. The additional fragment was absent from about 35% of the cells in this culture.

Table 7.3. Data on eleven primary urine cell cultures from which chromosome preparations were made in the minimum time.

Means of Collection*	Number	Days to chromosome result	
		Mean	Range
(i)	7	13.6	10-17
(ii)	4	15.5	14-17
(iii)	-	-	-
Total	11	14.1	10-17

* See table II, appendix II.

were of little clinical importance only eleven cultures were harvested for such studies in the minimum time. These eleven were not a random sample but were from cultures which were growing well. The data on these cultures are shown in table 7.3. There is little point in comparing these times statistically with those for amniotic fluid cultures. However, it can be seen that the times required for cytogenetic results from either type of culture are of the same magnitude.

Of the 46 successful primary urine cell cultures 5 degenerated and could not be subcultured. Hence 41 primary cultures were subcultured and data relating to these are shown in table 7.4. The time in primary culture was independent of the means of collection of the urines. The difference in this parameter between the

Table 7.4. Data relating to the 46 successful primary urine cell cultures.

Means of collection	Number	Primary culture degenerated	Days in primary culture
(i)	16	-	26.5 ± 10.49[a] 16 - 48[b]
(ii)	24	3	29.8 ± 8.05 19 - 50
(iii)	6	2	35.5 ± 13.58 21 - 53
Total	46	5	29.0 ± 9.72 16 - 53

a - mean ± standard deviation

b - range

urines collected via urethra and those collected at post-mortem is not significant. The urines collected by suprapubic aspiration from living children took longer in primary culture but the numbers involved (only four cases) are too small for this finding to be significant. The time in primary culture for the 41 urine cell strains was not different from that for the two series of amniotic fluid cell strains (see chapter 3).

The cell strains derived from the primary culture of urine cells suffered badly in the outbreaks of microbial and mycoplasma contamination. Only 12 cell strains were cultured to senescence without encountering such problems and data on these are shown in table 7.5. Any conclusions drawn from these data would be greatly influenced by the exclusion of the majority of the cell strains. The two cell strains showing the greatest growth potential (66 was discarded in the 35th passage and 137 in the 27th) both suffered from mycoplasma contamination and seven other cell strains which survived for more than 10 passages had to be discarded because of contamination. In view of this there is little point in carrying out statistical comparisons of the behaviour of these cell strains with that of amniotic fluid cell strains. Ten of the twelve cell strains were tested and found not to contain mycoplasma on at least one occasion, the other two were in culture at a time when no other cell strains yielded mycoplasma.

Of the 12 cell strains cultured to senescence there were 6 collected into urine collecting bags, 4 were

Table 7.5. Data pertaining to twelve urine cell strains cultured to senescence.

Laboratory number	Days in 1° culture	Number of passages	Days as cell strain	Mean interval between subcultures (days)
52	18	12	111	9.2
68	42	8	62	7.8
77	35	8	70	8.8
93	26	14	94	6.7
100	18	19	98	5.2
104	33	18	90	5.0
116	53	14	60	4.3
131	30	4	31	7.8
147	25	11	111	10.1
150	31	3	34	11.3
184	35	14	104	7.4
265	34	7	61	8.7
Mean	31.67	11.00	77.17	7.69
Standard Deviation	9.75	5.12	28.16	2.12

collected at post-mortem and 2 by suprapubic aspiration from living infants. The age range of the infants from whom the urines were collected was from one day to 23 days, however 7 were one day old and only one was older than one week. All had normal karyotypes except 116 (Edward's syndrome); there were 7 males and 5 females. The behaviour of the cell strains was apparently unrelated to any of these factors.

The data were examined for any apparent relationship

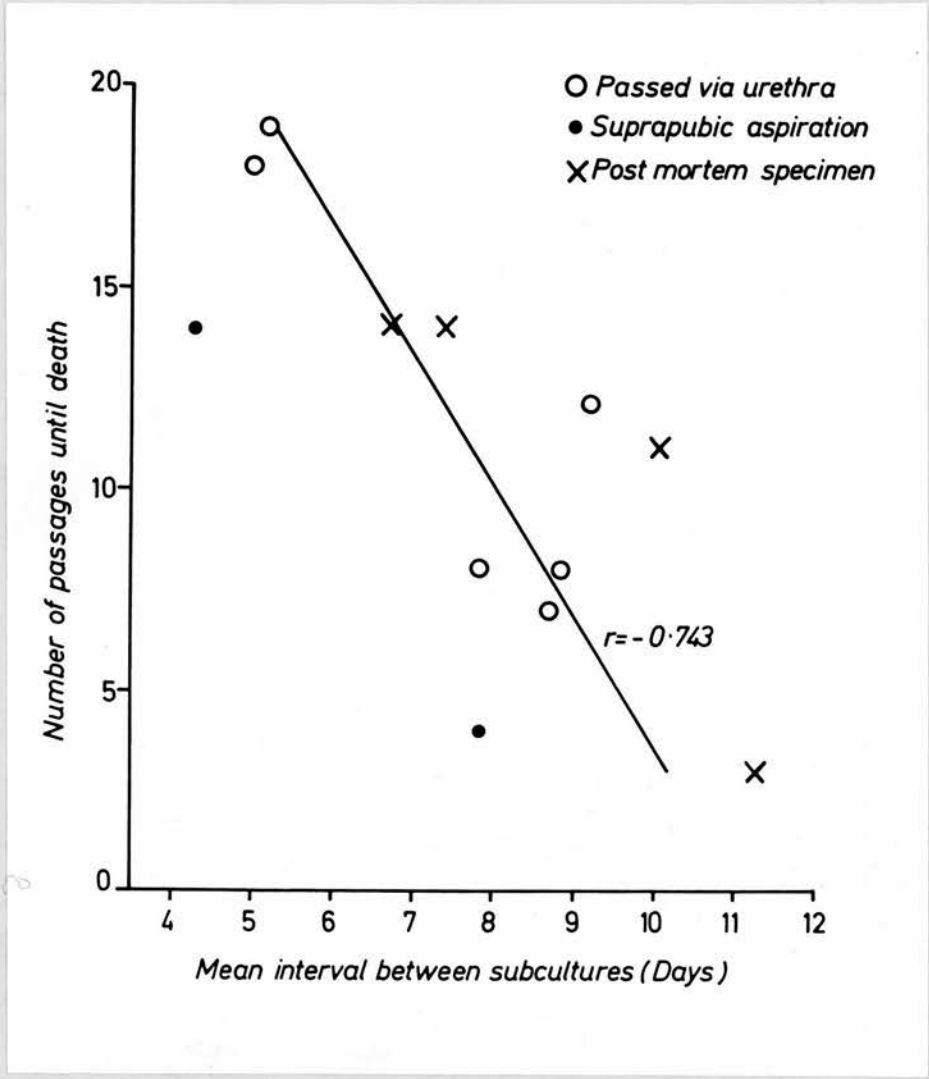
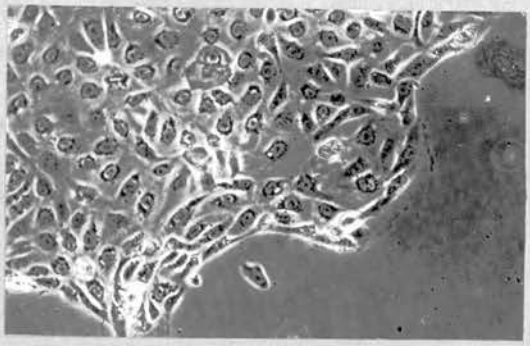


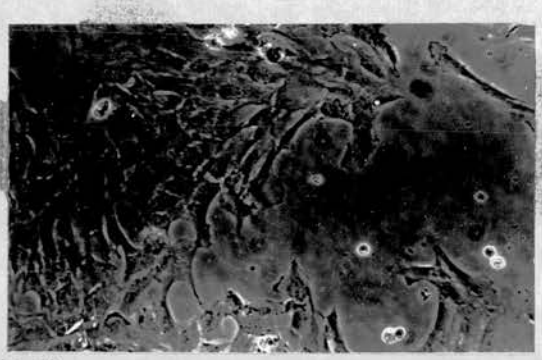
Fig. 7.7. Relationship between the number of passages for which urine cell strains could be cultured prior to death and the mean interval between subcultures.

between the various parameters of cell behaviour. As in the case of the amniotic fluid cell strains the only significant finding was a correlation between the number of passages and the mean interval between subcultures (Fig. 7.7). This correlation ($r = -0.743$) is highly significant ($P < .01$) and is of the same magnitude as that found for both series of amniotic fluid cell strains.

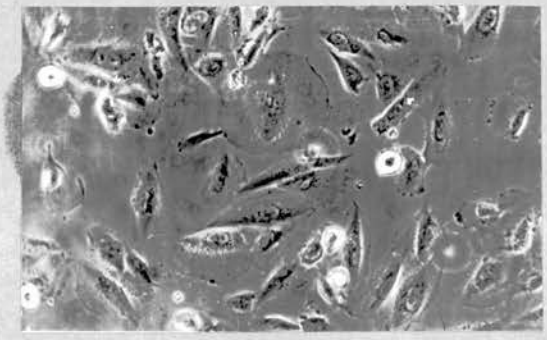
Most of the urine cell strains which survived for more than 4 or 5 passages were stored in the liquid nitrogen unit. Eight of these were removed from storage



(i)



(ii)



(iii)

(iv)

Fig. 7.8. Proliferating cells in primary urine cell cultures. (Phase contrast, x 100).

(i) Epithelioid type I cells, note well defined border of cell colony.
(ii) Epithelioid type III cells, note that there is no well defined border to the cell colony.
(iii) Epithelioid type II cells, note large size of the cells, fibrillar nature of the cytoplasm and "loose" nature of the cell colony.
(iv) Area of fibroblast-like cells.

and put back into culture; none were again cultured to senescence.

Three of the cell strains in this series (of 12) had cytogenetic studies performed after the 10th passage and examination of at least 10 cells revealed no departure from normal diploidy. A further 5 cell strains outwith this series had similar cytogenetic examinations and again no departure from normal diploidy was observed. The two cell strains in which *in vitro* chromosome changes, associated with mycoplasma contamination, occurred have been described in chapter 3.

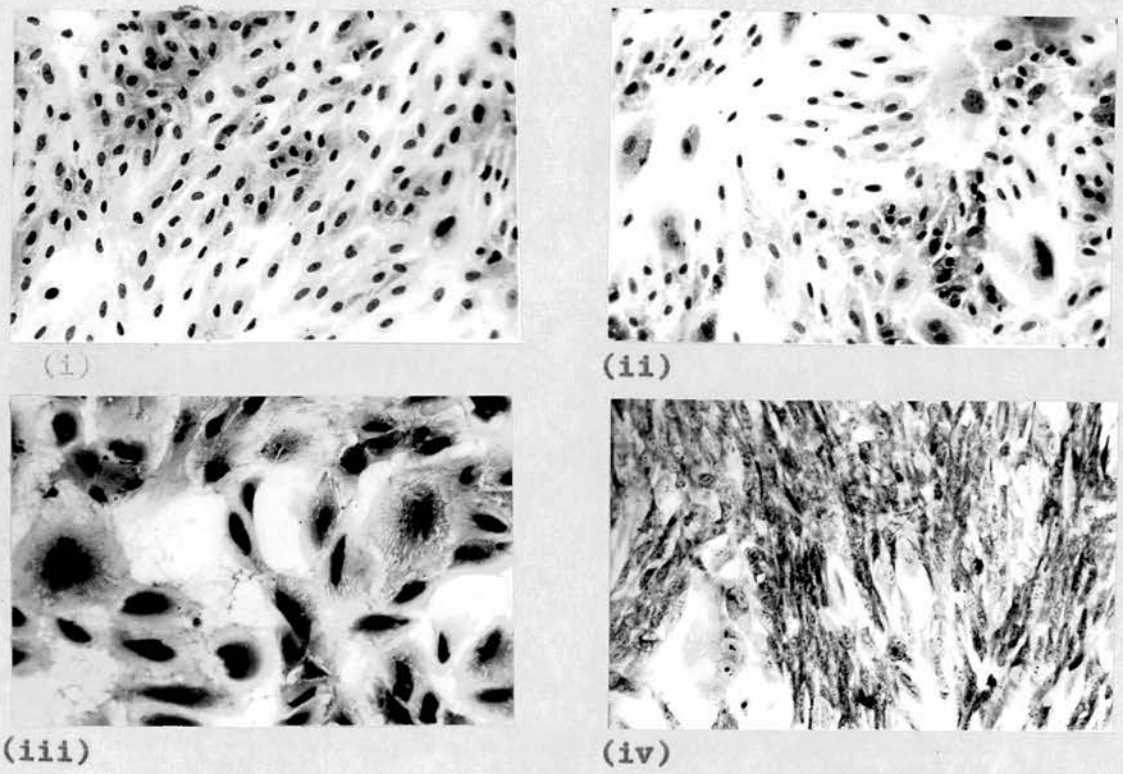


Fig. 7.9. Cells on flying coverslips prepared from urine cell strains (Stain, Giemsa, x 100).

(i) Epithelioid type III cells from second passage of culture.
(ii) Epithelioid type III cells with some type II cells from second passage of culture.
(iii) Epithelioid type II cells from sixth passage of culture, note variation in size with some binucleate cells.
(iv) Cell strain in 12th passage of culture showing epithelioid type II and III cells interspaced amongst fibroblast-like cells.

The morphology of the urine cell strains was indistinguishable from that of amniotic fluid cell strains under the light microscope. The only difference was that macrophages were less frequently seen in primary cultures. Typical examples of the morphology of cultured urine cells in primary culture and after serial cultivation are shown in Figs. 7.8 and 7.9.

Since the early passages were less affected by contamination (two cell strains were lost after the first subculture) than the later passages it was possible to

Table 7.6. Data on 39 cell strains cultured until there were enough cells for a biochemical assay.

Means of collection	Number	Days in 1° culture	Days to biochemical result
(i)	14	25.9 ± 11.11[a] 16 - 48[b]	36.8 ± 10.64 23 - 56
(ii)	21	29.8 ± 8.05 19 - 50	41.1 ± 10.42 25 - 64
(iii)	4	35.5 ± 13.58 21 - 53	45.0 ± 14.31 33 - 63
Total	39	28.9 ± 9.96 16 - 53	39.9 ± 10.9 23 - 64

a - mean ± standard deviation
b - range

study the time required to produce enough cells for a biochemical assay. This is of little clinical relevance in the post-natal situation, but as a parameter of cell strain behaviour it can be validly compared with that for amniotic fluid cell strains. The results of this study are shown in table 7.6. The differences between the times taken to achieve a biochemical result for the three groups of cell strains (according to method of collection of the urine) are not significant. As in the case of amniotic fluid cultures (see chapter 3) it can be seen that about two-thirds of the time required to produce a biochemical result is due to the time in primary culture.

The distribution of the times taken to achieve a biochemical result for the 39 cell strains is shown in Fig. 7.10. A comparison of these results with those for

the two similar series of amniotic fluid cell strains is shown in table 7.7. The differences between the two series of amniotic fluid cell strains and the urine cell strains are minimal and not significant.

The levels of 9 lysosomal enzymes in urine cell strains at the third passage were compared with those in amniotic fluid cell strains at the same stage of culture and with connective tissue derived fibroblast-like cell strains in early passages of culture. The results of this comparison are shown in table 7.8. Inspection of

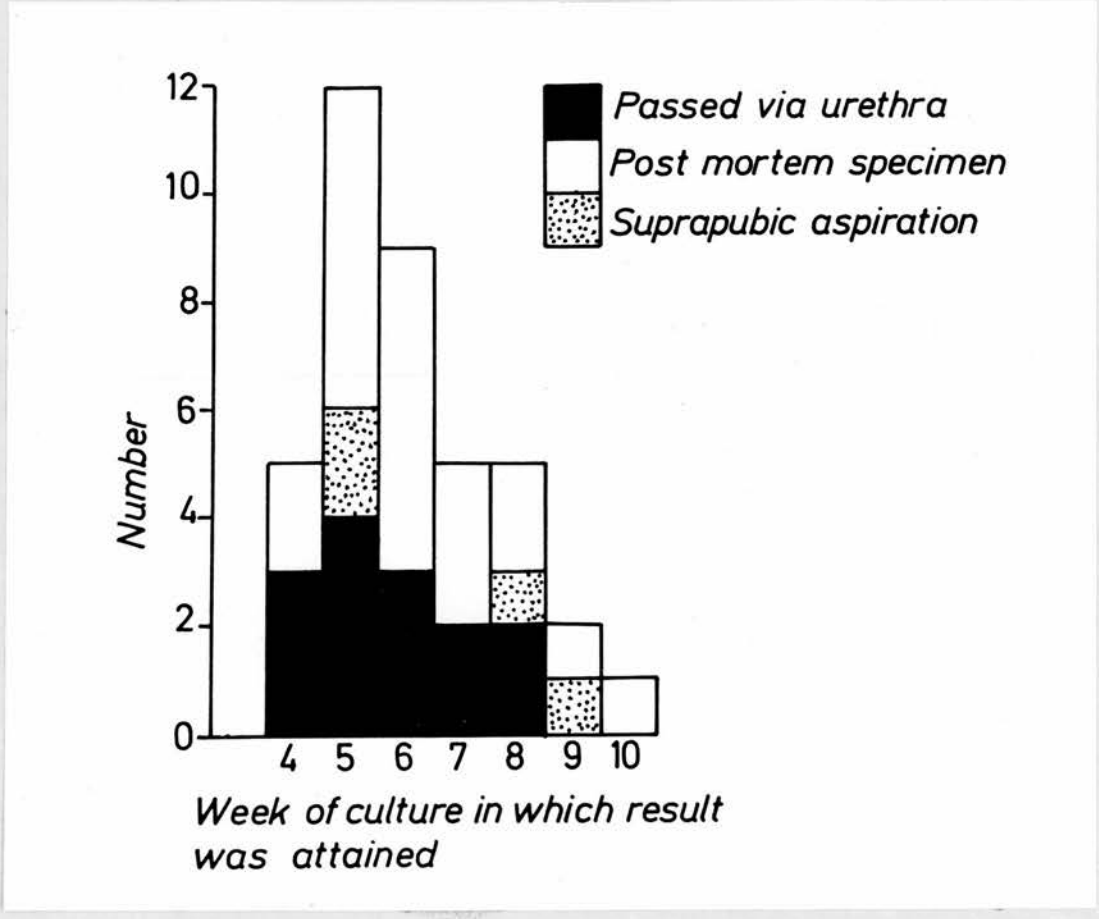


Fig. 7.10. Distribution of the times taken to achieve a biochemical result from 39 urine cell strains.

Table 7.7. Comparison of the times taken to achieve a biochemical result from amniotic fluid and urine cell cultures.

	Amniotic fluid		Urine
	First year	Second year	
Mean time to result (days)	41.4	37.2	39.9
Number successful	56	44	39
Success at 6 weeks	36 (64%)	32 (73%)	26 (67%)
Success at 8 weeks	49 (88%)	40 (91%)	36 (92%)

Table 7.8. Specific activities* of 9 lysosomal enzymes in amniotic fluid, urine and fibroblast-like cell strains.

Enzyme	Amniotic fluid[a]	Urine (n = 9)	Fibroblast-like (n = 20)
N-acetyl-ß-D-glucosaminidase	52.1 ± 16.7[b] 28.7 - 91.9[c]	52.7 ± 16.9 31.2 - 84.0	68.1 ± 29.5[d] 42.6 - 155.2
Acid Phosphatase	7.7 ± 2.8 4.3 - 12.7	7.3 ± 2.4 4.4 - 13.0	8.8 ± 2.7 3.6 - 13.9
ß-galactosidase	4.6 ± 2.0 2.4 - 10.7	4.8 ± 1.5 2.5 - 6.4	7.6 ± 2.5 3.3 - 12.8
ß-glucuronidase	1.30 ± 0.52 0.62 - 2.02	1.25 ± 0.54 0.72 - 2.49	1.12 ± 0.62 0.33 - 2.56[e]
ß-glucosidase	0.072 ± 0.030 0.030 - 0.170	0.071 ± 0.030 0.022 - 0.106	0.068 ± .047 0.025 - 0.200
α-galactosidase	0.42 ± 0.15 0.22 - 0.78	0.41 ± 0.088 0.27 - 0.55	0.50 ± 0.17 0.25 - 0.80
α-arabinosidase	0.53 ± 0.25 0.22 - 1.30	0.44 ± .103 0.32 - 0.68	0.44 ± .158 0.20 - 0.77
α-glucosidase	0.38 ± 0.18 0.17 - 0.97	0.40 ± 0.15 0.20 - 0.71	0.59 ± 0.29 0.22 - 1.32
α-mannosidase	0.65 ± 0.30 0.28 - 1.27	0.55 ± 0.19 0.37 - 0.85	0.99 ± 0.40 0.56 - 2.01

a Data from Butterworth et al. (1973a) appendix III.
b mean ± standard deviation; c range;
d n = 18; e n = 12
* nmole methylumbelliferone/min./mg.protein.

this table shows that the specific activities of the enzymes studied are very similar in the urine and amniotic fluid cell strains. The greatest differences are in the activities of α-arabinosidase and α-mannosidase and these are not statistically significant.

Comparison of activities of the enzymes in the three types of cell strains as shown in table 7.8 indicates that there are no differences for ß-glucuronidase, ß-glucosidase and α-arabinosidase. The differences between the activities are not statistically significant for acid phosphatase or α-galactosidase. The Student's t-test values for the remaining enzymes are shown in table 7.9. Hence it can be seen that the activities of α-mannosidase and ß-galactosidase are lower in urine and amniotic fluid cell strains than in fibroblast-like cell strains. The position is less well defined for N-acetyl-ß-D-glucosaminidase and α-glucosidase. In both cases the enzyme activities are very significantly lower in amniotic fluid cell strains than in fibroblast-like cell strains. However, these enzyme levels in urine cell strains, although virtually identical to those in the amniotic fluid cell strains, are not significantly different from those in fibroblast-like cell strains. This is probably due to the small number of urine cell strains studied and it is likely that further studies would indicate that N-acetyl-ß-D-glucosaminidase and α-glucosidase would be less active in urine cell strains than in fibroblast-like cell strains.

Table 7.9. Results of Student's t-tests on the difference in the activities of 4 enzymes in amniotic fluid and urine cell strains compared with fibroblast-like cell strains.

Enzyme	t-test values	
	Fibroblast vs. Urine	Fibroblast vs. Amniotic Fluid
N-acetyl-ß-D-glucosaminidase	$t_{25} = 1.443$ (N.S.)	$t_{55} = 2.613$ ($P < .01$)
ß-galactosidase	$t_{27} = 3.118$ ($P < .01$)	$t_{59} = 5.078$ ($P < .001$)
α-glucosidase	$t_{27} = 1.825$ (N.S.)	$t_{58} = 3.434$ ($P < .01$)
α-mannosidase	$t_{27} = 3.142$ ($P < .01$)	$t_{57} = 3.681$ ($P < .001$)

The enzyme levels were measured in one cell strain at daily intervals after subculture and the results are shown in table 7.10. Many of the enzymes show great variation in activity with time after subculture and these variations do not appear to follow any pattern. For some enzymes the highest level of activity seen was two or three times that of the lowest.

Table 7.10. Specific activities* of nine lysosomal enzymes in a urine cell strain assayed at varying times after subculture at the 10th passage.

Enzyme	Days after subculture				
	1	2	3	4	7
N-acetyl-ß-D-glucosaminidase	55.1	50.8	67.3	51.3	45.8
Acid Phosphatase	7.01	6.68	10.7	5.85	9.85
ß-galactosidase	2.67	2.51	3.01	1.84	2.67
ß-glucuronidase	2.19	0.91	1.05	0.74	1.19
ß-glucosidase	0.84	0.52	0.60	0.37	0.65
α-galactosidase	0.33	0.30	0.43	0.34	0.33
α-arabinosidase	0.43	0.36	0.46	0.35	0.35
α-glucosidase	0.21	0.16	0.19	0.12	0.15
α-mannosidase	1.59	1.25	1.46	1.16	0.81

* nmole methylumbelliferone/min./mg.protein.

Table 7.11. Specific activities* of nine lysosomal enzymes in one urine cell strain assayed at different passages.

Enzyme	Passage 1	2	3	4	5	7	9
N-acetyl-ß-D-glucosaminidase	26.1	41.4	27.6	29.9	26.6	42.0	33.0
Acid Phosphatase	5.60	4.15	3.78	4.48	3.58	4.77	3.57
ß-galactosidase	2.50	2.90	2.13	1.70	1.11	2.03	1.75
ß-glucuronidase	0.72	0.68	0.55	0.62	0.38	0.63	0.59
ß-glucosidase	0.033	0.067	0.035	0.051	0.046	0.086	0.035
α-galactosidase	0.24	0.39	0.19	0.25	0.23	0.30	0.27
α-arabinosidase	0.50	0.44	0.27	0.26	0.17	0.23	0.26
α-glucosidase	0.23	0.41	0.23	0.21	0.12	0.17	0.25
α-mannosidase	0.59	0.64	0.39	0.45	0.49	0.61	0.53

* nmole methylumbelliferone/min./mg. protein.

The variations in enzyme activity with increasing passage number were studied in one urine cell strain. The results are shown in table 7.11. For most of the enzymes the highest values are 2 or 3 times the lower ones. There do not appear to be any patterns in the changing activities of these enzymes related to passage of culture.

A urine cell strain was established from only one child with an inborn error of lysosomal enzyme metabolism. This was a two-year-old child (128) with Tay-Sachs disease. Enzyme studies (Dr. R.W. Tateson) indicated that the inborn error of metabolism was indeed reflected in the urine cell strain. The results of these studies are shown in table 7.12. The isoenzymes were separated by DEAE cellulose absorption and in this system Tay-Sachs

Table 7.12. N-acetyl-ß-D-glucosaminidase findings on a urine cell strain from a case of Tay-Sachs disease.

Cells assayed	N-acetyl-ß-D-glucosaminidase*		
	A	B	A/B
Normal urine	17.6	13.4	1.3
Tay-Sachs urine	3.5	25.6	0.14
Tay-Sachs skin fibroblasts	5.3	60.1	0.09

* arbitrary units

disease is defined as having an N-acetyl-ß-D-glucosaminidase A/B ratio of less than 0.5 whereas a value between 1 and 2 is considered normal.

DISCUSSION

The hypothesis that amniotic fluid cells which proliferate in culture are in part derived from the fetal urinary tract will be explored.

The rate of urine production by fetuses of gestational age from 30 to 40 weeks has been directly measured by an ultrasonic technique (Campbell et al., 1973; Wladimiroff and Campbell, 1974). At 30 weeks this was found to have a mean value of 9.6 ml./hour. Since the fetal kidney functions from the 20th week, and possibly from as early as the ninth (Emery, 1970) there is no reason to doubt that fetal urine is being excreted into the amniotic fluid from the end of the first trimester until term. Indeed Abramovich (1968) has estimated that at 11 weeks gestation the fetus produces 1.2 ml./day of urine. The finding of oligohydramnios in most cases of renal or genito-urinary malformations which prevent fetal micturition (Bain and Scott, 1960) suggests that the fetal kidneys may be a major source of amniotic fluid, especially in the second half of pregnancy.

It is known that urine from neonates is rich in cells for the first few days of life (Aas, 1961; Baccichetti et al., 1971). The cellular content of amniotic fluid shows a marked increase at about 13 to 17 weeks of gestation (Nelson and Emery, 1970). Hence shortly after the time at which the fetal kidney is almost certain to be functioning there is a marked rise in the cellular content of the amniotic fluid. The

ability of urine cells to proliferate in tissue culture has been confirmed (Bolande, personal communication; Dallaire,1973).

What arguments are there against this hypothesis? Obviously amniotic fluid in the early weeks of pregnancy, before the fetal kidney is functioning, cannot come from this source. The acellular component of the amniotic fluid is not all of fetal origin as a number of proteins have been shown to be of maternal origin (see Emery, 1973, for references) although others (e.g. the enzyme N-acetyl-ß-D-glucosaminidase) are certainly fetal. In early pregnancy the cellular content of amniotic fluid is low - Wachtel et al. (1969) even claimed that amniotic fluid prior to 16 weeks gestation was virtually acellular. In a study of the cytology of amniotic fluid Casadei et al. (1973) reported the presence of cells from fetal skin, oral mucosa and amniotic epithelium. They did not identify cells which they could attribute to the fetal urinary tract but all the amniotic fluids studied were of less than 20 weeks gestation. On the other hand Huisjes (1968) considered that the 'parabasal' cells of amniotic fluid were from the fetal bladder.

Hence circumstantial evidence suggests that fetal urine is one possible source of the cells in amniotic fluid. What of the experimental evidence? The only recorded experimental approach to this problem was by Jacobson and Barter (1967). They attempted to culture urine, yolk sac fluid, chorionic fluid and tracheal aspirates from human fetuses. Only the tracheal

aspirates produced cell growth. This does not however provide evidence as to the nature of the cells in the aspirate since the fetus carries out respiratory movements which cause amniotic fluid to travel in and out of the lungs (Dawes, 1973). Hence tracheal aspirate is mainly amniotic fluid, although as term approaches the concentration of total phospholipids, lecithin and chloride in pharyngeal fluid is approximately ten times that in the amniotic fluid (Biggs et al., 1974).

The ability of cells from urine to grow in culture implicates them as a source of the cells cultivated from amniotic fluid. Although it was not possible in the present study to obtain much fetal material, one bladder aspirate from an 18 week fetus grew well in culture. It should however be noted that when urine is collected by aspiration the needle has to pass through tissue and cellular contamination from this source is always a possibility, even if a remote one. The fact that urine collected by suprapubic aspiration can contain large numbers of cells refutes the claim by Biasini et al. (1969) that the cells in the urine of neonates come mainly from the terminal urethra. That urine cells are not the only ones in amniotic fluid which can proliferate in culture is demonstrated by the successful culture of two amniotic fluids of less than 8 weeks gestation (Jacobson and Barter, 1967), presumably prior to fetal kidney function and micturition.

One problem associated with urine cell culture which

requires explanation is that of the very poor success rates in culture compared with amniotic fluid. Much of this can probably be attributed to the nature of the fluid in which these cells occur. Amniotic fluid is maintained at a constant pH (Cassady and Barnett, 1969) and the osmolarity is fairly constant although it does fall towards term (Cassady and Barnett, 1968; Lind et al., 1969). In other words, cells in amniotic fluid are in an isotonic medium which shows little variation in pH. In addition toxic wastes such as uric acid and creatinine are maintained at low levels until near term (Jakabovits et al., 1972; Harrison, 1972). On the other hand cells in urine are in a fluid which varies greatly in pH and osmolarity and whose concentration of waste products is higher than in amniotic fluid - such conditions can do little to maintain cell viability. Hence the differences in success in culturing cells from two such fluids is hardly surprising. Indeed the diminished success rate in culturing cells from amniotic fluids collected at term (see chapter 2) may well be due to this increase in waste product concentration in the amniotic fluid at that time.

When the cells from urine had been successfully cultured, there were no discernable differences between these and amniotic fluid cells in either behaviour in culture or morphology. This morphological similarity, especially the presence of epithelioid type II cells (see chapter 4) indicates that these cells do not necessarily arise from the chorionic villi as suggested by Uhlendorf

(1970).

The findings from the enzyme studies are of particular interest. Comparison of these with amniotic fluid cell strains and connective tissue derived fibroblast-like cell strains indicates that the urine cell strains are similar in their lysosomal enzyme patterns to amniotic fluid cell strains. In the case of ß-galactosidase and α-mannosidase urine and amniotic fluid cell strains have lower activities than do fibroblasts and the same is probably the case for α-glucosidase and N-acetyl-ß-D-glucosaminidase.

Kaback and Leonard (1970) compared the levels of three lysosomal enzymes in amniotic fluid cell strains with cultured maternal and fetal fibroblast-like cells. Their findings are not strictly comparable with those of the present study. However they did find that ß-galactosidase and N-acetyl-ß-D-glucosaminidase levels in cultured amniotic fluid cells were higher than those in fetal fibroblast-like cells. This was also the case for a comparison with maternal fibroblast-like cells for ß-galactosidase but not for N-acetyl-ß-D-glucosaminidase. In the case of arylsulphatase A, not included in the present study, the levels in amniotic fluid cell strains were found to be intermediate between those in maternal and fetal fibroblast-like cells. Leroy et al. (1973) published data similar, in part, to that of Kaback and Leonard (1970). They found no difference in the activity of the 3 enzymes studied by Kaback and Leonard (1970) when comparing amniotic fluid cell strains and

fibroblast-like cells (presumably not of fetal origin). O'Brien et al. (1972) found that N-acetyl-β-D-glucosaminidase A activity was higher in cultured amniotic fluid cells than in cultured skin fibroblast-like cells. Possible reasons for these different findings from different laboratories are the lack of standardised procedures for cell homogenisation (see chapter 6) and enzyme assay. Kaback and Leonard (1970) used p-nitrophenol conjugates as substrates rather than the methylumbelliferone conjugates used in the present study and by O'Brien et al. (1972).

The findings of marked variations in lysosomal enzyme levels in urine cell strains with time factors associated with tissue culture are very similar to those found in amniotic fluid cell strains (see chapter 6). This in itself does not indicate that the lysosomal enzyme biochemistry of urine cell strains is more like that of amniotic fluid cell strains than that of fibroblast-like cell strains. Such studies were not carried out using fibroblast-like cell strains but lysosomal enzyme levels have been reported to show great variation in these cell strains (Hultberg et al., 1973).

The enzyme which might have been of greatest use in demonstrating a similarity between urine and amniotic fluid cell strains and a difference between these and fibroblast-like cell strains was histidase. This enzyme was reported (Melancon et al., 1971) to be present in epitheloid but not fibroblast-like cells derived from amniotic fluid. Attempts to assay this enzyme were

unfortunately unsuccessful (Dr. J. Butterworth). It was, however, later reported that histidase was not present in either skin fibroblast-like cells or cultured amniotic fluid cells which were epithelial in nature (Ota, 1973). This report documented difficulties in the enzyme assay akin to those found in the present study, and its results suggested that errors could be made such that non-specific changes in the enzyme reaction mixture could be interpreted as enzyme activity. The question of whether histidase activity is present in cultured amniotic fluid cells of one type and not in another remains open. However, in view of the report of Ota (1973) and work in the present study it is probable that this enzyme can not be assayed in cultured cells using currently available techniques.

Carter (1970) has written "Mistakes can be made if it is assumed that the enzyme levels in amniotic cells are necessarily the same as in fibroblast cultures". This study has shown that such an assumption would be invalid for some lysosomal enzymes. Kaback and Leonard (1970) have demonstrated that amniotic fluid cells have lower activities of arylsulphatase A than do skin fibroblast-like cells from adults although their findings are not supported by Leroy et al. (1973). Other differences between the activities of enzymes in amniotic fluid cell strains and in fibroblast-like cell strains have been reported (see Brock, 1973, for references).

In enzyme assays for the antenatal diagnosis of inborn errors of metabolism, two types of control material are required. The first is a normal control,

that is cells which do not have the inborn error of metabolism in question and hence have normal levels of the enzyme being assayed. The best such control would be amniotic fluid cells at the same stage of culture as those in which the enzyme levels are to be determined. If such material is not available then urine cells at the same stage of culture may well be a more suitable alternative to skin fibroblast-like cells which are another possibility. The second type of control material required is cultured cells which are known to have the inborn error of metabolism in question. Ideally these would be cultured amniotic fluid cells from a previously affected case in the same family. This sort of material will rarely be available hence skin fibroblast-like cells from a previously affected member of the family are regarded as the next best control material with skin fibroblast-like cells from a similarly affected individual outside the family in question being a possible third. It is suggested that in view of the similarity between cultured amniotic fluid and urine cells that the latter would be preferable as control material to skin fibroblast-like cells. This may well be of particular importance where the enzyme levels in a disease state are not totally absent but present in variable amounts up to about 10% of normal. Hence it would be desirable to produce urine cell strains from infants known to have inborn errors of metabolism and to store these in liquid nitrogen units as control material for future antenatal diagnoses.

SUMMARY

The culture of cells from the urine of newborn infants and fetuses is described. Urine cell culture was not as successful as amniotic fluid cell culture and factors such as age and gestation of the urine donor together with cellular content, osmolarity and acidity of the urine, which may affect success have been examined. The behaviour and morphology of cultured urine cells has been studied and compared with that of cultured amniotic fluid cells; no differences between these two types of cells in tissue culture could be demonstrated. The levels of activity of a group of lysosomal enzymes in urine cell strains were compared with those in amniotic fluid and connective tissue derived fibroblast-like cell strains. There were definite similarities between urine and amniotic fluid cell strains and these were different from fibroblast-like cell strains for ß-galactosidase and α-mannosidase. This was probably also the case for α-glucosidase and N-acetyl-ß-D-glucosaminidase. The inborn error of metabolism in Tay Sachs disease was expressed in cultured urine cells. The use of urine cell strains, in preference to skin fibroblast-like cell strains, as control material for the antenatal diagnosis of inborn errors of metabolism is suggested. The hypothesis that cells which can be cultivated from amniotic fluid are in part derived from the fetal urinary tract is explored.

Chapter 8

Conclusions

When this project was commenced the use of cultured amniotic fluid cells for the antenatal diagnosis of chromosome abnormalities was slowly coming into general use. In spite of this much remained to be learnt about the cytogenetics of amniotic fluid cultures; tetraploidy and aneuploidy, with its implications in the diagnosis of mosaicism, were ill understood. The antenatal diagnosis of inborn errors of metabolism was in its infancy, both from the biochemical and tissue culture aspects. There was virtually no data available on the culture of amniotic fluid cells for the antenatal diagnosis of these biochemical defects or on the behaviour of amniotic fluid cell strains in long-term serial cultivation. Apart from some studies on cell viability, from which no conclusions could be drawn, there had been little systematic study of the behaviour of primary cultures of amniotic fluid collected throughout pregnancy. The origin of the cells in amniotic fluid which proliferate in culture was the subject of speculation based only on cytological evidence. At the same time antenatal diagnosis of gross CNS lesions was not possible since the basic work by Brock and his colleagues had not been published.

What is the present situation? It is now possible to perform the antenatal diagnosis of gross CNS defects by estimation of amniotic fluid α-fetoprotein levels. The antenatal diagnosis of chromosome abnormalities is now fairly widespread with many laboratories offering this service. The biochemistry of many inborn errors

of metabolism is much better understood, hence the chances of making errors in these diagnoses should be diminished. Nevertheless, in most cases this aspect of antenatal diagnosis must still be regarded as experimental. Sophisticated microassay techniques have been developed which should enable the antenatal diagnosis of some inborn errors of metabolism within the same time as that required for chromosome abnormalities.

The present study has made significant contributions to the understanding of the behaviour of amniotic fluid cells in tissue culture. The success, judged by any criterion, of primary cultures of amniotic fluid was unrelated to gestational age or the reason for collection of the fluid except that fluids collected at induction of labour at term were difficult to cultivate. Similarly the time required to culture sufficient cells for a biochemical assay, and the behaviour of the cell strains in serial culture were not related to the origin of the amniotic fluid. Amniotic fluid cells are relatively easy to grow in tissue culture and variations in culture methods had little effect with the exception that the method of Gray et al. (1971) was found to be less successful than more conventional methods.

The morphology of the cell types which proliferate in tissue culture was studied and a classification of the cell types proposed. One cell type, the macrophage, was found to be present in greatly increased numbers in anencephalic amniotic fluids. Studies of the macrophages in such amniotic fluids and those from Rhesus isoimmunised

and normal pregnancies failed to reveal their origin. In anencephalic and spina bifida amniotic fluids the increase in the number of macrophages is probably associated with the open lesion. Amniotic fluids from Rhesus isoimmunised pregnancies have a slightly increased number of macrophages and this may well be associated with the increase in placental Hofbauer cells described in this condition. Although the findings of macrophages must await further studies for their full significance to be realised, they appear at present to offer an additional method for the antenatal diagnosis of gross CNS malformations.

Cytogenetic studies of primary amniotic fluid cultures have confirmed that technology in this field is reliable and suitable for screening pregnancies at risk. Apart from cases with a family history of chromosome abnormality the major group which should benefit comprises those women who become pregnant towards the end of their reproductive life. Although the major chromosome abnormality to be detected in such cases will be trisomy 21, other abnormalities will undoubtedly be detected such as the case of trisomy 18 diagnosed antenatally in this study. Some problems in the cytogenetics of amniotic fluid are discussed, including the interrelated problems of aneuploidy, <u>in vitro</u> chromosome change and the diagnosis of mosaicism. In all cases where fetal mosaicism has been diagnosed from amniotic fluid cultures, the diagnosis has not stood the test of extrauterine verification. Possible explanations of these cases of

misdiagnosis of mosaicism have included some form of *in vitro* chromosome change. In this study *in vitro* chromosome changes were demonstrated in cell strains but not in primary cultures. Unfortunately these changes were associated with mycoplasma contamination and the question of whether some amniotic fluid cell cultures can undergo spontaneous *in vitro* chromosome changes remains unresolved. Other cytogenetic studies, on cell strains not contaminated with mycoplasma, indicated that chromosomal stability, as seen in other human diploid cell strains, was a feature of amniotic fluid cell strains.

The finding that cells from the urine of neonates will proliferate in tissue culture suggested a possible site of origin for the amniotic fluid cells which proliferate in tissue culture. Studies of urine cell strains indicated that they were similar to amniotic fluid cell strains in all respects studied. Lysosomal enzyme patterns of the urine cell strains were more closely resembled by those of amniotic fluid cell strains than those of connective tissue derived fibroblast-like cell strains. The hypothesis that the cells which proliferate in amniotic fluid cultures are derived in part from the fetal urinary tract has been explored and evidence has been put forward to suggest that this hypothesis is correct.

The behaviour of a number of lysosomal enzymes in cultured amniotic fluid cell strains has been studied. The range of activity of the enzymes in normal amniotic

fluid cell strains was established for this laboratory. It was found that the enzyme activity in these cell strains showed marked fluctuations when measured at different stages of tissue culture. In spite of an extensive study of possible factors which could have been related to these fluctuations their source remained undetected. Similar fluctuations in the activity of lysosomal enzymes in fibroblast-like cell strains have been reported. The special significance of these fluctuations in the antenatal diagnosis of inborn errors of lysosomal enzyme metabolism has been discussed and it was concluded that the diagnosis of such a condition should never be based on the results obtained from assay of the cells from a single culture vessel.

Four pregnancies in which the fetus was at risk for an inborn error of metabolism were monitored. Two had favourable outcomes in that normal infants were predicted and delivered. Two pregnancies, at risk for maple-syrup-urine disease, from one woman were unsuccessfully monitored. The first was terminated after failure of the cell cultures and the second resulted in an affected infant after a normal infant had been predicted. This case demonstrated the folly of attempting antenatal diagnosis when the initial diagnosis on which the procedure is based is not at the enzyme level. The general problem of culturing sufficient cells in a reasonable period of time when monitoring such pregnancies is discussed and the results from the present study suggest that the chances of having sufficient cells within 6 weeks of amniotcentesis

are too low for this procedure to be offered in a clinical situation. This situation will change when microassay methods for the enzymes involved in inborn errors of metabolism are sufficiently developed to enable enzyme assays on primary cultures. Progress in this direction has been made by Galjaard et al. (1972, 1973) but their methods are highly specialised and not widely applicable at present.

In summary, the present study has resulted in a number of findings and conclusions.

1. Amniotic fluid cells are not difficult to cultivate in vitro.

2. The behaviour of amniotic fluid cells in primary and long-term culture has been documented.

3. Cytogenetic studies of amniotic fluid cultures provide a rapid, reliable and, within the constraints of any method involving cultured cells, straightforward means of establishing the fetal karyotype.

4. Amniotic fluid from pregnancies in which the fetus has a gross CNS malformation has an increased number of macrophages and this finding may form the basis of a method for the antenatal diagnosis of such fetuses.

5. The behaviour of a number of lysosomal enzymes in amniotic fluid has been documented, marked fluctuations in their activities have been described and possible reasons for them sought.

6. Cells from the urine of neonates can proliferate in tissue culture. This finding suggests a possible

source for some of the amniotic fluid cells which can be cultured.

It has been stated that "Genetics has the reputation among some practising clinicians of being concerned with rare, esoteric and untreatable disease ....." (Finn, 1973). If the present study has helped, even to a very small extent, to dispel such notions then it has been worthwhile.

Acknowledgements and Declaration

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I declare that, apart from the assistance acknowledged, I have carried out this work myself and that this thesis is of my own composition.

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Grant R. Sutherland.

References

Aas, K. 1961. The cellular excretion in the urine of normal newborn infants. Acta Paediat. Scand. 50, 361

Abbo, G., Zellweger, H. 1970. Prenatal determination of fetal sex and chromosomal complement. Lancet 1, 216

Abramovich, D.R. 1968. The volume of amniotic fluid in early pregnancy. J. Obstet. Gynaec. Br. Commonw. 75, 728

Arrighi, F.E., Hsu, T.C. 1971. Localization of heterochromatin in human chromosomes. Cytogenetics 10, 81

Aula, P., Nichols, W.W. 1967. The cytogenetic effects of mycoplasma in human leukocyte cultures. J. cell. Physiol. 70, 281

Baccichetti, C., Tenconi, R., Largaiolli, G. 1971. Screening test for Y chromosome in newborn using urinary epithelial cells. Lancet 11, 1328

Bain, A.D., Bowie, J.H., Flint, W.F., Beverley, J.K.A., Beattie, C.P. 1956. Congenital toxoplasmosis simulating haemolytic disease of the newborn. J. Obstet. Gynaec. Br. Emp. 63, 826

Bain, A.D., Scott, J.S. 1960. Renal agenesis and severe urinary tract dysplasia. A review of 50 cases, with particular reference to the associated anomalies. Br. med. J. 1, 841

Bain, A.D., Tateson, R., Anderson, J.M., Cumings, J.N. 1972. Sandhoff's disease (G_{M2} gangliosidosis, type 2) in a Scottish family. J. ment. Defic. Res. 16, 119

Bauld, R., Sutherland, G.R., Bain, A.D. 1974. Chromosome studies in the investigation of stillbirths and neonatal deaths. Arch. Dis. Child. (in press)

Beck, C., Tappel, A.L. 1968. Rat-liver lysosomal ß-glucosidase: a membrane enzyme. Biochim. biophys. Acta 151, 159

Berman, P.H., Balis, M.E., Dancis, J. 1969. A method for the prenatal diagnosis of congenital hyperuricemia. J. Pediat. 75, 488

Beutler, E., Kuhl, W., Trinidad, F., Teplitz, R., Nadler, H. 1971. ß-Glucosidase activity in fibroblasts from homozygotes and heterozygotes for Gaucher's disease. Am. J. Hum. Genet. 23, 62

Biasini, G., Rocchi, L., Montaguti, A. 1967. Il significato delle cellule epiteliali presenti nella urina del neonato. Riv. Clin. Pediat. 80, 442

Biggs, J.S.G., Hemming, J., McGeary, H., Gaffney, T.J. 1974. Human amniotic and fetal neonatal pharyngeal fluids. J. Obstet. Gynaec. Br. Commonw. 81, 70

Blakely, S.B. 1937. The diagnosis of the sex of the human fetus in utero. Am. J. Obstet. Gynec. 34, 322

Bonsnes, R.W. 1966. Composition of amniotic fluid. Clin. Obstet. Gynec. 9, 440

Bourne, G. 1962. The human amnion and chorion. Lloyd-Luke Ltd. London

Brady, R.O., Uhlendorf, B.W., Jacobson, C.B. 1971. Fabry's disease: antenatal detection. Science, N.Y. 172, 174

Brevis, D.C.A. 1950. Composition of liquor amnii in haemolytic disease of newborn. Lancet 11, 443

Brevis, D.C.A. 1952. The antenatal prediction of haemolytic disease of the newborn. Lancet 1, 395

Brock, D.J.H. 1972. Inborn errors of metabolism. In: The biochemical genetics of man. Brock, D.J.H. and Mayo, O., editors. Academic Press, London

Brock, D.J.H. 1973. Biochemical studies on amniotic fluid cells. In: Antenatal diagnosis of genetic disease. Emery, A.E.H., editor . Churchill Livingstone, Edinburgh and London

Brock, D.J.H., Sutcliffe, R.G. 1972. Alpha-fetoprotein in the antenatal diagnosis of anencephaly and spina bifida. Lancet 11, 197

Butler, L.J., Reiss, H.E. 1970. Antenatal detection of chromosome abnormalities. J. Obstet. Gynaec. Br. Commonw. 77, 902

Butler, L.J., Reiss, H.E., France, N.E., Briddon, S. 1973. Antenatal diagnosis of Patau's syndrome (trisomy 13) including a detailed pathological study of the fetus. J. med. Genet. 10, 367

Butterworth, J., Sutherland, G.R., Bain, A.D., McCrae, W.M. 1972. Lysosomal enzymes in amniotic fluid. Clin. Chim. Acta 39, 275

Butterworth, J., Broadhead, D.M., Sutherland, G.R., Bain, A.D. 1974. Lysosomal enzymes of amniotic fluid in relation to gestational age. Am. J. Obstet. Gynec. 'in press'

Campbell, S., Wladimiroff, J.W., Dewhurst, C.J. 1973. The antenatal measurement of fetal urine production. J. Obstet. Gynaec. Br. Commonw. 80, 680

Cantarow, A., Stuckert, H., Davis, R.C. 1933. The chemical composition of amniotic fluid. A comparative study of human amniotic fluid and maternal blood. Surg. Gynec. Obstet. 57, 63

Carter, C.O. 1970. Practical aspects of early diagnosis. In Early diagnosis of human genetic defects. Harris, M., editor. Fogarty international center proceedings No. 6

Casadei, R., D'Ablaing, G., Kaplan, B.J., Schwinn, C.P. 1973. A cytologic study of amniotic fluid. Acta Cytol. 17, 289

Caspersson, T., Farber, S., Foley, G.E., Kudynowski, J., Modest, E.J., Simonsson, E., Wagh, U., Zech, L. 1968. Chemical differentiation along metaphase chromosomes. Expl. Cell Res. 49, 219

Cassady, G., Barnett, R. 1968. Amniotic fluid electrolytes and perinatal outcome. Biol. Neonat. 13, 155

Cassady, G., Barnett, R. 1969. Acid-base and gas tension studies of the amniotic fluid in human gestation. Biol. Neonat. 14, 251

Cederqvist, L.L., Fuchs, F. 1970. Antenatal sex determination. A historical review. Clin. Obstet. Gynec. 13, 159

Cederqvist, L.L., Wennerstrom, C., Senterfit, L.B., Baldridge, P.B., Rothe, D.J. 1973. Simplified method for the accelerated growth of amniotic fluid cell cultures. Am. J. Obstet. Gynec. 116, 871

Chester, D.C., Penny, S.R., Emery, J.L. 1971. Fat containing macrophages in the cerebrospinal fluid of children with hydrocephalus. Dev. Med. Child Neurol. 13, Supp. 25, 33

Chitham, R.G., Quayle, S.J., Hill, L. 1973. The selection of a method for the culture of cells from amniotic fluid. J. clin. Path. 26, 721

Cockburn, F., Robins, S.P., Forfar, J.O. 1970. Free amino-acid concentrations in fetal fluids. Br. med. J. 3, 747

Court Brown, W.M., Buckton, K.E., Jacobs, P.A., Tough, I.M., Kuenssberg, E.V., Knox, J.D.E. 1966. Chromosome studies on adults. Eugenics Laboratory Memoirs XLII, Cambridge University Press, London

Dallaire, L., Melancon, S.B., Potier, M., Guay, P., Lamothe, P. 1973. Prognostic value of fetal cell and amniotic fluid studies in prenatal diagnosis. Paper presented 4th International Conference on Birth Defects, Vienna, September, 1973. Abstract: Excerpta Medica Int. Congr. Ser. No. 297, 78

Dancis, J. 1968. The antepartum diagnosis of genetic diseases. J. Pediat. 72, 301

Daniel, C. 1904. Recherches sur la cytologie du liquide amniotique. Ann. Gynec. 61, 466

Dawes, G.S. 1973. Revolutions and cyclical rhythms in prenatal life: fetal respiratory movements rediscovered. Pediatrics, Springfield 51, 965

Den Tandt, W.R., Schaberg, A. 1973. Mucopolysaccharide storage diseases and lysosomal hydrolases in cultured fibroblasts. Path. Europ. 8, 3

Desnick, R.J., Krivit, W., Sharp, H.L. 1973. In utero diagnosis of Sandhoff's disease. Biochem. Biophys. Res. Commun. 51, 20

Drets, M.E., Shaw, M.W. 1971. Specific banding patterns of human chromosomes. Proc. Nat. Acad. Sci., U.S.A. 68, 2073

Eagle, H. 1973. The effect of environmental pH on the growth of normal and malignant cells. J. cell. Physiol. 82, 1

Emery, A.E.H. 1970. Antenatal diagnosis of genetic disease. In Modern trends in human genetics-1. Emery, A.E.H., editor. Butterworths. London

Emery, A.E.H. 1973. Biochemical analysis of amniotic fluid. In Antenatal diagnosis of genetic disease. Emery, A.E.H., editor. Churchill Livingstone. Edinburgh and London

Emery, A.E.H., Burt, D., Nelson, M.M., Scrimgeour, J.B. 1970. Antenatal diagnosis and aminoacid composition of amniotic fluid. Lancet 1, 1307

Epstein, C.J., Schneider, E.L., Conte, F.A., Friedman, S. 1972. Prenatal detection of genetic disorders. Am. J. hum. Genet. 24, 214

Ferguson-Smith, M.E., Ferguson-Smith, M.A., Nevin, N.C., Stone, M. 1971. Chromosome analysis before birth and its value in genetic counselling. Br. med. J. 4, 69

Finn, R. 1973. Review of Burnet, F.M. (1972) Autoimmunity and auto-immune disease. J. med. Genet. 10, 99

Fitzgerald, P.H., Brehaut, L.A. 1970. Depression of DNA synthesis and mitotic index by colchicine in cultured human lymphocytes. Expl. Cell Res. 59, 27

Fluharty, A.L., Scott, M.L., Porter, M.T., Kihara, H., Wilson, M.G., Towner, J.W. 1973. Acid α-glucosidase in amniotic fluid. Biochem. Med. 7, 39

Fogh, J., Fogh, H. 1965. Chromosome changes in PPLO-infected FL human amnion cells. Proc. Soc. exp. Biol. Med. 119, 233

Fox, H. 1967. The incidence and significance of Hofbauer cells in the mature human placenta. J. Path. Bact. 93, 710

Fuchs, F. 1960. Genetiske undersøgelser af fostre in utero. Nord. Med. 64, 1490

Fuchs, F., Philip, J. 1963. Mulighed for antenatal undersøgelse af fosterets kromosomer. (Abstract) Nord. Med. 69, 572

Fuchs, F., Riis, P. 1960. Kønsbestemmelse på fostret før afgørelse af eugenisk abortindikation. Nord. Med. 64, 1481

Fujimoto, W.Y., Seegmiller, J.E., Uhlendorf, B.W., Jacobson, C.B. 1968. Biochemical diagnosis of an X-linked disease in utero. Lancet 11, 511

Galjaard, H., Fernandes, J., Jahodova, M., Koster, J.F., Niermeijer, M.F. 1972. Prenatal diagnosis of genetic disease. Bull. Europ. Soc. Hum. Genet. Nov., 79

Galjaard, H., Mekes, M., De Josselin De Jong, J.E., Niermeijer, M.F. 1973. A method for rapid prenatal diagnosis of glycogenosis II (Pompe's disease). Clin. Chim. Acta 49, 361

Gerbie, A.B., Melancon, S.B., Ryan, C., Nadler, H.L. 1972. Cultivated epithelial-like cells and fibroblasts from amniotic fluid: Their relationship to enzymatic and cytologic analysis. Am. J. Obstet. Gynec. 114, 314

Gerbie, A.B., Nadler, H.L., Gerbie, M.V. 1971. Amniocentesis in genetic counselling. Am. J. Obstet. Gynec. 109, 765

Golde, D.W., Byers, L.A., Finley, T.N. 1974. Proliferative capacity of human alveolar macrophage. Nature 247, 373

Gray, C., Davidson, R.G., Cohen, M.M. 1971. A simplified technique for the culture of amniotic fluid cells. J. Pediat. 79, 119

Greene, A.E., Toji, L., Nichols, W.W., Coriell, L.L. 1973. Amniocentesis cells: culture, cryogenic storage, isozymes, and finite life in vitro. In Vitro 9, 156

Greenfield, J.G., Carmichael, E.A. 1925. The cerebro-spinal fluid in clinical diagnosis. MacMillan and Co., London.

Habeshaw, J.A. 1970. Determinants of phagocytosis: a study of the variable factors involved in the interaction between red blood cells and macrophages. Ph.D. Thesis, University of Edinburgh

Ham, R.G. 1963. An improved nutrient solution for diploid Chinese hampster and human cell lines. Expl. Cell Res. 29, 515

Hamerton, J.L. 1970. Fetal sex. Lancet 1, 516

Hamerton, J.L. 1971. Human cytogenetics I. General cytogenetics. Academic press. New York and London

Harrison, R.F. 1972. Amniotic fluid uric acid levels in the maturing fetus. J. Obstet. Gynaec. Br. Commonw. 79, 708

Hay, R.J. 1970. Cell strain senescence in vitro: Cell culture anomaly or an expression of a fundamental inability of normal cells to survive and proliferate. In Aging in cell and tissue culture. Holeckova, E., Cristofalo, V.J., editors. Plenum Press. New York. London

Hayflick, L. 1965. The limited in vitro lifetime of human diploid cell strains. Expl. Cell Res. 37, 614

Hayflick, L., Moorhead, P.S. 1961. The serial cultivation of human diploid cell strains. Expl. Cell Res. 25, 585

Ho, M.W., O'Brien, J.S., Radin, N.S., Erickson, J.S. 1973. Glucocerebrosidase: Reconstitution of activity from macromolecular components. Biochem. J. 131, 173

Hofbauer, J. 1903. Ueber das kostante Vorkommen bisher unbekannter zelliger Formelemente in der Chorionzotte der menschlichen Plazenta. Klin. Wschr. 16, 871

Hsu, L.Y.F., Dubin, E.C., Kerenyi, T., Hirschhorn, K. 1973. Results and pitfalls in prenatal cytogenetic diagnosis. J. med. Genet. 10, 112

Hsu, L.Y.F., Strauss, L., Dubin, E., Hirschhorn, K. 1973a. Prenatal diagnosis of trisomy 18. Am. J. Dis. Child. 125, 290

Hughes, D.T., Rink, E., Griffiths, S., Paintin, D.B. 1971. Fetal karyotype from amniotic cells without culturing. Lancet 11, 1319

Huisjes, H.J. 1968. Amniotic fluid cytology. Van Gorcum and Co., Netherlands

Hultberg, B., Sjöblad, S., Öckerman, P.A. 1973. Properties of five acid hydrolases in human skin fibroblast cultures. Acta Paediat. Scand. 62, 474

Jacobson, C.B. 1965. The cytogenetics of spontaneous abortion. Ob Gyn Observer 4, 6

Jacobson, C.B., Barter, R.H. 1967. Intrauterine diagnosis and management of genetic defects. Am. J. Obstet. Gynec. 99, 796

Jacoby, F. 1965. Macrophages. In Cells and tissues in culture. Methods, biology and physiology, 2. Willmer, E.N., editor. Academic press. London, New York.

Jacoby, L.B., Littlefield, J.W., Milunsky, A., Shih, V.E., Wilroy, R.S. 1972. A microassay for argininosuccinase in cultured cells. Am. J. Hum. Genet. 24, 321

Jacobovits, A., Farkas, M., Piukovich, I. 1972. Cytologic patterns and creatinine concentration of the amniotic fluid. Acta Cytol. 16, 381

Jeffcoate, T.N.A., Fliegner, J.R.H., Russell, S.H., Davis, J.C., Wade, A.P. 1965. Diagnosis of the adrenogenital syndrome before birth. Lancet 11, 553

Kaback, M.M., Howell, R.R. 1970. Infantile metachromatic leukodystrophy. Heterozygote detection in skin fibroblasts and possible applications to intrauterine diagnosis. New Engl. J. Med. 282, 1336

Kabak, M.M., Leonard, C.O. 1970. Control studies in the antenatal diagnosis of human genetic metabolic disease. In Early diagnosis of human genetic defects. Harris, M., editor. Fogarty international center proceedings No. 6

Kaback, M.M., Leonard, C.O. 1972. Morphological and enzymological considerations in antenatal diagnosis. In Antenatal diagnosis. Dorfman, A., editor. University of Chicago Press

Kajii, T. 1971. Pseudomosaicism in cultured amniotic-fluid cells. Lancet 11, 1037

Kanfer, J.N., Spielvogel, C. 1972. ß-Galactosidase activity of human skin fibroblasts. Biochim. biophys. Acta 289, 359

Kardon, N.B., Chernay, P., Hsu, L.Y., Martin, J.L., Hirschhorn, K. 1972a. Pitfalls in prenatal diagnosis resulting from chromosomal mosaicism. J. Pediat. 80, 297

Kardon, N.B., Chernay, P.R., Hsu, L.Y.F., Martin, J.L., Hirschhorn, K. 1972b. Problems in prenatal diagnosis resulting from chromosomal mosaicism. Clin. Genet. 3, 83

Klinger, H.P. 1965. Personal communication to Steele and Breg (1966)

Knörr-Gärtner, H., Härle, I. 1972. A modified method of culturing human amniotic fluid cells for prenatal detection of genetic disorders. Humangenetik 14, 333

Kohn, G., Robinson, A. 1970. Tetraploidy in cells cultured from amniotic fluid. Lancet 11, 778

Kundsin, R.B., Ampola, M., Streeter, S., Neurath, P. 1971. Chromosomal aberrations induced by T strain mycoplasmas. J. med. Genet. 8, 181

Kundsin, R.B., Driscoll, S.G., Ming, P.M.L. 1967. Strain of mycoplasma associated with human reproductive failure. Science, N.Y. 157, 1573

Lee, C.L.Y., Gregson, N.M., Walker, S. 1970. Eliminating red blood-cells from amniotic-fluid samples. Lancet 11, 316

Lee, J.C., Bendel, R.P., Brooker, D.C. 1972. Aneuploidy in cultured amniotic cells. J. Am. med. Ass. 219, 1211

Leroy, J.G., Van Elsen, A.F., Martin, J.-J., Dumon, J.E., Hulet, A.E., Okada, S., Navarro, C. 1973. Infantile metachromatic leukodystrophy. Confirmation of a prenatal diagnosis. New Engl. J. Med. 288, 1365

Liley, A.W. 1961. Liquor amnii analysis in the management of the pregnancy complicated by rhesus sensitization. Am. J. Obstet. Gynec. 82, 1359

Lind, T., Parkin, F.M., Cheyne, G.A. 1969. Biochemical and cytological changes in liquor amnii with advancing gestation. J. Obstet. Gynaec. Br. Commonw. 76, 673

Lisgar, F., Gertner, M., Cherry, S., Hsu, L.Y., Hirschhorn, K. 1970. Prenatal chromosome analysis. Nature 225, 280

Littlefield, J.W. 1971. Problems in the use of cultured amniotic fluid cells for biochemical diagnoses. Birth Defects Orig. Art. Ser. 7, 15

Litwin, J. 1970. Standardization of human diploid cell cultivation. Appl. Microbiol. 20, 899

Litwin, J. 1972. Human diploid cell response to variations in relative amino acid concentrations in Eagle medium. Expl. Cell Res. 72, 566

Lowden, J.A., Cutz, E., Conen, P.E., Rudd, N., Doran, T.A. 1973. Prenatal diagnosis of G_{M1}-gangliosidosis. New Engl. J. Med. 288, 225

Machin, G.A. 1974. Chromosome abnormality and perinatal death. Lancet 1, 549

Macintyre, M.N. 1971. Chromosomal problems of intra-uterine diagnosis. Birth Defects: Orig. Art. Ser. 5, 10

Makepeace, A.W., Fremont-Smith, F., Dailey, M.E., Carroll, M.P. 1931. The nature of the amniotic fluid. A comparative study of human amniotic fluid and maternal serum. Surg. Gynec. Obstet. 53, 635

McDougall, J.K. 1971. Adenovirus-induced chromosome aberrations in human cells. J. gen. Virol, 12, 43

Medawar, P.B. 1957. The uniqueness of the individual. Methuen and Co. London

Melancon, S.B., Lee, S.Y., Nadler, H.L. 1971. Histidase activity in cultivated human amniotic fluid cells. Science, N.Y. 173, 627

Miles, C.P. 1964. Prolonged culture of diploid human cells. Cancer Res. 24, 1070

Milunsky, A. 1973. The prenatal diagnosis of hereditary disorders. Charles C. Thomas. Springfield

Milunsky, A., Atkins, L., Littlefield, J.W. 1972. Amniocentesis for prenatal genetic studies. Obstet. Gynec. 40, 104

Milunsky, A., Spielvogel, C., Kanfer, J.N. 1972a. Lysosomal enzyme variations in cultured normal skin fibroblasts. Life Sciences 11, (2), 1101

Mintzer, R.A., Reisman, L.E. 1969. Prenatal chromosome studies. Diagnosis in genetic defects. Sthn. med. J. 62, 1220

Moore, G.E., McLimans, W.F. 1968. The life span of the cultured normal cell: Concepts derived from studies of human lymphoblasts. J. Theoret. Biol. 20, 217

Moorhead, P.S., Nowell, P.C., Mellman, W.J., Battips, D.M., Hungerford, D.A. 1960. Chromosome preparations of leukocytes cultured from human peripheral blood. Expl. Cell Res. 20, 613

Morison, J.E. 1963. Foetal and neonatal pathology. Butterworths, London

Morrow, G., Schwarz, R.H., Hallock, J.A., Barness, L.A. 1970. Prenatal detection of methylmalonic acidemia. J. Pediat. 77, 120

Mulcahy, M.T., Jenkyn, J. 1973. Prenatal diagnosis. Results of cytogenetic analysis of amniotic fluid cell cultures. Med. J. Aust. 1, 979

Nadler, H.L. 1968a. Antenatal detection of hereditary disorders. Pediatrics, Springfield 42, 912

Nadler, H.L. 1968b. Patterns of enzyme development utilizing cultivated human fetal cells derived from amniotic fluid. Biochem. Genet. 2, 119

Nadler, H.L. 1969. Prenatal detection of genetic defects. J. Pediat. 74, 132

Nadler, H.L. 1971. Indications for amniocentesis in the early prenatal detection of genetic disorders. Birth Defects Orig. Art. Ser. 7, 5

Nadler, H.L., Egan, T.J. 1970. Deficiency of lysosomal acid phosphatase. A new familial metabolic disorder. New Engl. J. Med. 282, 302

Nadler, H.L., Gerbie, A.B. 1969. Enzymes in non-cultured amniotic fluid cells. Am. J. Obstet. Gynec. 103, 710

Nadler, H.L., Gerbie, A.B. 1970. Role of amniocentesis in the intrauterine detection of genetic disorders. New Engl. J. Med. 282, 596

Nadler, H.L., Gerbie, A. 1971. Present status of amniocentesis in intrauterine diagnosis of genetic defects. Obstet. Gynecol. 38, 789

Nadler, H.L., Messina, A.M. 1969. In-utero detection of type-II glycogenosis (Pompe's disease). Lancet II, 1277

Nakagome, Y., Iinuma, K., Matsunaga, E. 1972. Polyploidy in cultured amniotic-fluid cells. Lancet II, 387

Nelson, M.M. 1973a. Antenatal sex determination. In Antenatal diagnosis of genetic disease. Emery, A.E.H., editor. Churchill Livingstone. Edinburgh and London

Nelson, M.M. 1973b. Laboratory methods of use in antenatal diagnosis. Ibid.

Nelson, M.M., Emery, A.E.H. 1970. Amniotic fluid cells; prenatal sex prediction and culture. Br. med. J. 1, 523

Nelson, M.M., Emery, A.E.H. 1973. Amniotic fluid cell cultures. J. med. Genet. 10, 19

Nelson, M.M., Ruttiman, M.-T., Brock, D.J.H. 1974. Predictive value of amniotic-fluid macrophages in gross C.N.S. defects. Lancet 1, 504

O'Brien, J.S., Okada, S., Fillerup, D.L., Veath, M.L., Adornato, B., Brenner, P.H. 1972. Tay-Sachs disease: prenatal diagnosis. In Antenatal diagnosis. Dorfman, A., editor. University of Chicago Press

O'Brien, J.S., Okada, S., Fillerup, D.L., Veath, M.L., Adornato, B., Brenner, P.H., Leroy, J.G. 1971. Tay-Sachs disease: prenatal diagnosis. Science, N.Y. 172, 61

Ota, D.M. 1973. The use of histidase for the study of amniotic cell origin and histidinemia. Am. J. Obstet. Gynec. 117, 567

Paris Conference, 1971. Standardization in human cytogenetics. Birth Defects: Orig. Art. Ser. VIII: 7, 1972. The National Foundation, New York

Parrish, H.M., Rountree, M.E., Lock, F.R. 1958. Technique and experience with transabdominal amniocentesis in 50 normal patients. Am. J. Obstet. Gynec. 75, 724

Paul, J. 1965. Cell and tissue culture. Livingstone. Edinburgh and London

Potter, E.L. 1961. Pathology of the fetus and infant. Second edition. Year book medical publishers, Inc., Chicago

Prescott, G.H., Pernoll, M.L., Hecht, F., Nicholas, A. 1973. A prenatal diagnosis clinic: an initial report. Am. J. Obstet. Gynec. 116, 942

Prochownick, L. 1877. Beiträge zur Lehre vom Fruchtwasser und seiner Entstehung. Archiv Gynaecol. 11, 304

Rask-Madsen, J., Philip, J. 1970. The chromosome complement of human endometrium. Cytogenetics 9, 24

Ratcliffe, S.G., Keay, A.J. 1973. Chromosome studies on 11,000 newborn infants. (Abstract). Arch. Dis. Child, 48, 407

Rees, G.O. 1838. Chemical examination of the liquor amnii. Guy's Hospital Reports. 3, 393

Riis, P., Fuchs, F. 1960. Antenatal determination of foetal sex in prevention of hereditary diseases. Lancet 11, 180

Riis, P., Fuchs, F. 1966. Sex chromatin in cells of amniotic fluid and antenatal sex diagnosis. In The sex chromatin. Moore, K.L., editor. Philadelphia, W.B. Saunders.

Robertson, J.G. 1969. Management of patients with Rh isoimmunisation based on amniotic fluid examination. Am. J. Obstet. Gynec. 103, 713

Robinson, A., Bowes, W., Droegemueller, W., Puck, M., Goodman, S., Shikes, R., Greenshur, A. 1973. Intrauterine diagnosis: potential complications. Am. J. Obstet. Gynec. 116, 937

Ryan, C.A., Lee, S.Y., Nadler, H.L. 1972. Effect of culture conditions on enzyme activities in cultivated human fibroblasts. Expl. Cell Res. 71, 388

Saksela, E., Moorhead, P.S. 1963. Aneuploidy in the degenerative phase of serial cultivation of human cell strains. Proc. natn. Acad. Sci. U.S.A. 50, 390

Santesson, B., Akesson, H.O., Böök, J.A., Brosset, A. 1969. Karyotyping human amniotic-fluid cells. Lancet 11, 1067

Schneck, L., Friedland, J., Valenti, C., Adachi, M., Amsterdam, D., Volk, B.W. 1970. Prenatal diagnosis of Tay-Sachs disease. Lancet 1, 582

Scrimgeour, J.B. 1973. Amniocentesis: Technique and complications. In Antenatal diagnosis of genetic disease. Emery, A.E.H., editor. Churchill Livingstone. Edinburgh and London

Shrewsbury, J.F.D. 1933. Observations on the chemistry of the liquor amnii. Lancet 1, 415

Sly, W.S., Quinton, B.A., McAlister, W.H., Rimoin, D.L. 1973. Beta glucuronidase deficiency: report of clinical, radiologic and biochemical features of a new mucopolysaccharidosis. J. Pediat. 82, 249

Stanbridge, E., Önen, M., Perkins, F.T., Hayflick, L. 1969. Karyological and morphological characteristics of human diploid cell strain WI-38 infected with mycoplasmas. Expl. Cell Res. 57, 397

Steele, M.W., Breg, W.R. 1966. Chromosome analysis of human amniotic-fluid cells. Lancet 1, 383

Stuart, A.E. 1967. Isolation and culture of human macrophages from the living and the dead. J. Path. Bact. 93, 338

Stuart, A.E., Davidson, A.E. 1971. The human reticular cell: morphology and cytochemistry. J. Path. 103, 41

Sumner, A.T., Evans, H.J., Buckland, R.A. 1971. New technique for distinguishing between human chromosomes. Nature New Biology 232, 31

Sutcliffe, R.G., Brock, D.J.H. 1971. Enzymes in uncultured amniotic fluid cells. Clin. Chim. Acta 31, 363

Sutcliffe, R.G., Brock, D.J.H., Robertson, J.G., Scrimgeour, J.B., Monaghan, J.M. 1972. Enzymes in amniotic fluid: A study of specific activity patterns during pregnancy. J. Obstet. Gynaec. Br. Commonw. 79, 895

Sutherland, G.R. 1970. Chromosome abnormalities in newborn babies. Aust. J. Ment. Ret. 1, 77

Sutherland, G.R., Bauld, R., Bain, A.D. 1973. Chromosome studies and the paediatric post-mortem. Paper read to the 19th annual meeting of the Paediatric Pathology Society, Glasgow, October 1973

Sutherland, G.R., Bauld, R., Bain, A.D. 1974. Chromosome abnormality and perinatal death. Lancet 1, 752

Tappel, A.L. 1969. Lysosomal enzymes and other components. In Lysosomes in biology and pathology. Dingle, J.R., Fell, H.B., editors. North-holland, Amsterdam, London

Taussig, F.J. 1927. The amniotic fluid and its quantitative variability. Am. J. Obstet. Gynec. 14, 505

Therkelsen, A.J., Petersen, G.B., Steenstrup, O.R., Jonasson, J., Lindsten, J., Zech, L. 1972. Prenatal diagnosis of chromosome abnormalities. Acta Paediat. Scand. 61, 397

Thiede, H.A., Creasman, W.T., Metcalfe, S. 1966. Antenatal analysis of the human chromosomes. Am. J. Obstet. Gynec. 94, 589

Tjio, J.H., Levan, A. 1956. The chromosome number of man. Hereditas 42, 1

Todaro, G.J., Green, H. 1964. Serum albumin supplemented medium for long term cultivation of mammalian fibroblast strains. Proc. Soc. exp. Biol. Med. 116, 688

Turnbull, A.C., Gregory, P.J., Laurence, K.M. 1973. Antenatal diagnosis of fetal abnormality with special reference to amniocentesis. Proc. R. Soc. Med. 66, 1115

Uhlendorf, B.W. 1970. Use of amniotic fluid and reliability of diagnostic procedures. In Early diagnosis of human genetic defects. Harris, M., editor. Fogarty international center proceedings No.6

Uhlendorf, B.W., Mudd, S.H. 1968. Cystathionine synthase in tissue culture derived from human skin: enzyme defect in homocystinuria. Science, N.Y. 160, 1007

Uyeno, D. 1919. The physical properties and chemical composition of human amniotic fluid. J. Biol. Chem. 37, 77

Valenti, C., Kehaty, T. 1969. Culture of cells obtained by amniocentesis. J. Lab. clin. Med. 73, 355

Valenti, C., Schutta, E.J., Kehaty, T. 1969. Cytogenetic diagnosis of Down's syndrome in utero. J. Am. med. Ass. 207, 1513

Wachtel, E., Gordon, H., Olsen, E. 1969. Cytology of amniotic fluid. J. Obstet. Gynaec. Br. Commonw. 76, 596

Walker, S., Gregson, N.M., Hibbard, B.M. 1971. Nuclear sex and antenatal diagnosis of Klinefelter's syndrome. Lancet 11, 430

Walker, S., Lee, C.L.Y., Gregson, N.M. 1970. Polyploidy in cells cultured from amniotic fluid. Lancet 11, 1137

Wallace, D.C. 1972. Birth defects: A geneticist's review of current concepts. Aust. N.Z. J. Obstet. Gynaec. 12, 110

Wendl, U., Rudiger, H.W., Passarge, E., Mikkelsen, M. 1973. Maple syrup urine disease: rapid prenatal diagnosis by enzyme assay. Humangenetik 19, 127

Whitfield, C.R., Chan, W.H., Sproule, W.B., Stewart, A.D. 1972. Amniotic fluid lecithin: sphingomyelin ratio and fetal lung development. Br. med. J. 2, 85

Williams, J.W. 1930. Obstetrics. Sixth edition. D. Appleton and Co. New York, London

Wladimiroff, J.W., Campbell, S. 1974. Fetal urine-production rates in normal and complicated pregnancy. Lancet 1, 151

Woollard, H.H. 1924. Vital staining of the leptomeninges. J. Anat. 58, 89

Yoshida, M.C., Makino, S. 1963. A chromosome study of non-treated and an irradiated human in vitro cell line. Jap. J. Hum. Genet. 8, 39

Zur Hausen, H. 1967. Induction of specific chromosomal aberrations by adenovirus type 12 in human embryonic kidney cells. J. Virol. 1, 1174

Appendix I

Materials and methods used for tissue culture

This appendix contains details of the types of tissue culture vessels used and the methods of cleaning them. Details of the tissue culture medium and other solutions used for tissue culture are also included. Methods used in tissue culture in this study, but not recorded elsewhere are outlined.

GLASSWARE

The following glass items were used during this study:

1. Baby feeding bottles, 125 ml capacity, approximately 20 cm^2 growth surface area (Fig. A1.1).
2. Baby feeding bottles, 225 ml capacity, approximately 40 cm^2 growth surface area (Fig. A1.1).
3. Roux flasks, 1 litre capacity, approximately 240 cm^2 growth surface area.
4. Centrifuge tubes, 15 ml capacity, conical base.
5. Coverslips 6 x 22 mm, No. 1 (Chance Ltd.).

The culture vessels were all cleaned using the following procedure:

(a) rinsed in cold water;

(b) placed in hot water to which 1-2% Dri-Decon (Decon Laboratories Ltd.) had been added;

(c) cleaned on a mechanically rotating bottle brush;

(d) transferred to "Gallay Labmatic" glassware washing machine where they were washed in hot water and rinsed several times with cold water. The last two rinses were in deionised water.

Fig. A1.1. Main culture vessels used throughout this study. Plastic (Nunclon) Petri dish, 50 mm. (left); baby feeding bottle, 125 ml. capacity (middle); baby feeding bottle, 250 ml. capacity (right).

(e) dried, capped and then sterilised in hot air at 160°C for two hours.

Centrifuge tubes were similarly washed and siliconised monthly with 2% dimethyldichlorosilane in CCl_4 ("Repelcote", Hopkins and Williams Ltd.).

Coverslips were boiled in a 5% solution of Dri-Decon for 10 min., rinsed well in tap water then boiled in two changes of deionised water. They were stored in absolute ethanol and when required were removed from the alcohol, polished between two linen covered blocks, placed in a glass Petri dish and sterilised in hot air.

DISPOSABLE PLASTICS

Two types of disposable plastic culture vessels were

used:

1. Nunclon-delta Petri dishes, 50 mm diameter, 13 mm deep with three air vents (Fig. A1.1).

2. Nunclon-delta T-flasks, 30 ml capacity 25 cm^2 growth surface area.

TISSUE CULTURE MEDIA AND SOLUTIONS

The tissue culture medium used for almost all this work was Ham's F10 (Ham, 1963) which was prepared from the following components:

(a) Ham's F10 concentrate (10x) (Flow Laboratories Ltd.).

(b) Deionised water.

(c) L-glutamine, 200 mM solution prepared from BDH Chemicals Ltd. reagent.

(d) Fetal (bovine) calf serum (Biocult Laboratories Ltd.).

(e) Kanamycin sulphate B.P.C. ("Kanasyn", Winthrop Laboratories) or penicillin-streptomycin ("Crystamycin", Glaxo Laboratories).

(f) 4.4% $NaHCO_3$ solution prepared from "Analar" analytical reagent.

The medium was prepared using non-sterile deionised water. As soon as the medium had been prepared, ultra-filtration was carried out by passing it through a 142 mm. diameter Millipore filter with a pore size of 0.22 μ. The filtered medium was sterility tested by inoculation into Robertson's cooked meat medium which was plated on to blood agar after 16 hours incubation.

Other solutions used for tissue culture were:

(i) Trypsin solution, 0.25% in PBS prepared from Trypsin 1:250 (Difco Laboratories) and sterilised by the same filtration procedure as used for the tissue culture medium.

(ii) Phosphate buffered saline (PBS) prepared from "Oxoid" tablets and sterilised by autoclaving at 15 p.s.i. for 20 minutes. When the full "Dulbecco A" PBS was required a Ca/Mg solution was added.

INCUBATION OF PRIMARY AMNIOTIC FLUID CULTURE

Primary cultures were set up in open Petri dishes, hence incubation in an atmosphere of 5% CO_2 in air was required. This was achieved by carrying out incubation in an anaerobic jar. A rack was constructed, from

Fig. A1.2. Incubation system used for Petri dishes during this study. Anaerobic jar and rack constructed from acrylic sheeting.

acrylic sheeting, to fit inside the jar (Fig. A1.2). This rack could hold 36 of the Petri dishes used. The Petri dishes were placed on the rack in the jar, the lid secured and the gas cocks opened. The jar was then flushed for about 5 minutes with 5% CO_2 in air, the cocks closed and the jar placed in an incubator at 37°C. The only modification of the jar required was the removal of the catalyst and sealing of the side arm. The anaerobic jars used were manufactured by Baird and Tatlock to a modified McIntosh and Fildes design.

FLYING COVERSLIPS

Flying coverslips were prepared regularly from most cell strains for morphological studies. The technique of preparation, staining and fixation is after Paul (1965). Coverslips (6 x 22 mm) were placed in 6 x $\frac{3}{8}$" test tubes. To each tube was added 1 to 2 ml. of cell suspension. The tubes were incubated at 5° from horizontal until the cells on the coverslip became confluent. The fixation and staining procedure was as follows:

(1) The medium was removed from the tube.

(2) The coverslip was washed twice in PBS (Ca and Mg enriched).

(3) The coverslip was fixed for 5 min. in two changes of absolute methanol.

(4) The coverslip was air-dried and transferred to a staining jar.

(5) The coverslip was stained for 5 min. in Giemsa (Gurr's "R66") diluted 1 in 10 with buffer pH 7 (Gurr's

buffer tablets).

(6) The coverslip was rinsed twice in buffer pH 7.0 and then twice in deionised water, air dried and mounted on a slide.

COUNTING CELLS IN AMNIOTIC FLUID

Cells were counted in an "improved Neubauer" cell counting chamber. The total inscribed area of this chamber contains a volume of 0.9 µl. Hence the cell count of the amniotic fluid (cells/ml.) is the number of cells in the inscribed area x 10/9 x 1000. The amniotic fluid was not diluted but introduced directly into the chamber. Since the presence of only 1 cell in the inscribed area would give a cell count of approximately 1100 cells/ml. this must be regarded as the lower limit of sensitivity of this method. Amniotic fluids with one or no cells within the inscribed area were given an arbitrary cell count of 1000 cells/ml. Urine cell counts were also performed using this method and those with very low cell counts were also given an arbitrary count of 1000 cells/ml.

Appendix II

Data on amniotic fluids and urines studied

This appendix contains data on the amniotic fluids and urines included in this study. The data is set out in four tables:

Table I contains data pertaining to all the amniotic fluids used in this study over the two year period 1.12.71 to 30.11.73.

Table II contains data pertaining to the urines used in the study where proliferating cells were seen in the primary cultures. The urines for which no growth was seen in primary culture have been omitted.

Table III contains data on all the pregnancies monitored diagnostically by antenatal chromosome studies over the period 1.12.71 to 31.1.74.

Table IV contains data on all amniotic fluids on which macrophage counts were carried out.

It should be noted that the sample numbers are not consecutive since other material, not included in this study, was incorporated within the laboratory numbering system.

Table I. Data on all amniotic fluids studied over the two year period 1.12.71 to 30.11.73.

1	2	3	4	5	6	7	8	9	10	11	12
36	ii	12		23	+	*	29	8	34	43	
37	ii	12		16	+		22	6	30	37	
38	ii	15		49	+	*	49	4	63	30	j
40	ii	17		25	+		38		48		
42	iv	15				*			0		b
43	ii	15			0	N			0		b
44	i	32		33	+	*	33		47		i5
45	i	25		26	+		25		33		i6
46	i	35		26	+		34		48		i5
47	ii	18		20	+		33	5	43	34	1
48	ii	16		18	+	*	26	4	39	19	
49	ii	17		22	+	*	23		34		i6
50	i	23		21	+	*	23	5	30	31	
55	ii	18		13	+		18	14	29	135	
57	i	24		12	+	*	29	9	53	83	
59	ii	23	51	16	+		35	11	50	76	1
62	ii	22	145		+	*	28	12	45	107	k42; 1
63	i	32		15	+	*	30	11	47	80	1
64	i	31				N	30		0		h1
69	ii	16			+		29	11	40	77	1
70	ii	22	65		+		26	25	36		f; 1
71	i	22			+	*	28	11	49	106	e; 1
72	i	31			+		26	25	36	146	1
73	i	18			+		28	18	36	144	1
74	i	23			+		26	18	42	125	1
75	i	34			+		26	17	39	127	1

Table I (cont'd.)

1	2	3	4	5	6	7	8	9	10	11	12
76	i	30				*	26		0		h1
81	i	26			+	*	41	4	61	42	k71
82	i	26			+		26	22	36	129	k74; 1
83	i	34			+		26	22	39	137	1
84	i	34			+	*	26	14	34	90	k76; 1
85	i	27			+		26	23	34	111	1
86	i	22		26	+		24	9	41	108	k73; 1
89	i	27		12	+	*	29	18	41	110	1
90	i	28		14	+		19	10	25	71	k74; 1
92	i	24		12	+		20	15	34	110	k73; 1
94	ii	18	3.3	15	+	*	25	20	35	114	1
108	vi	41			0	N			0		a
109	vi	39		19	+	*	27	11	37	84	1
110	vi	38			+	*	32		43		i4
111	i	34		26	+	*	27		39		g19; k89
115	iii	15	10	19	+		30		35		f; i28; 1
118	ii	12	4.5	14	+		20	16	32	89	1
119	i	34		14	+		20	29	32	115	1
120	i	31		19	+		20	11	32	75	1
121	i	34		14	+	*	20	14	27	82	1
122	ii	16		15	+	*	21		34		g17
123	i	33		21	+		49	3	74	35	k86; 1
124	ii	11		19	+		33	15	46	69	1
127	i	35		19	+		36	17	42	69	k86; 1
129	ii	16	13.3	14	+	*			0		b
136	i	19		7	+	*	31		37		i12; 1

Table I (cont'd.)

1	2	3	4	5	6	7	8	9	10	11	12
152	i	24	110		+	*	34	13	44	109	1
168	vi	40	75			N	28	2	35	26	1
169	vi	39	1000		O				O		a
171	i	30		13	+	*	20	7	31	64	k136
176	i	20		17	+	*	20	35	28	142	1
177	i	36			+	*	34	8	60	103	
179	i	23				*	23	8	46	115	1
180	i	33		26	+	*			O		b; k136
181	i	30			+	*	27		43		i9; k152
186	ii	22		16	+		20		34		i5
189	i	23	95	21	+	*	28	9	41	84	k176
190	i	25	200		+		24	55	36	191	1
191	i	20	45	12	+		43	2	69	25	
192	i	32	65	14	+	*	43	9	64	78	k152
195	i	26	158	14	+	*	41	8	62	72	k179; 1
200	iii	35	33	16	+	*	30	10	57	115	1
202	i	22					46	6	64	67	1
203	i	26		13	+	*	31	11	46	98	k176
205	iv	15	5		+		26		38		i4
206	i	26			+		55	8	69	71	k202
207	i	30			+				O		d; e
209	i	31		17	+		37		46		h9; k190
210	i	29				*	36		43		h9; k176; 1
213	i	27		12	+		30		46		h10; k191
214	i	31				N			O		c
216	iii	16	14.5	19	+				O		d

Table I (cont'd.)

1	2	3	4	5	6	7	8	9	10	11	12
217	iii	16	5						0		b
220	iii	14	1	38	+				0		d
224	i	33				*	30		41		h9; k176; l
225	i	30					33	16	46	82	k191; l
226	iii	16	17	14	+				0		d
227	iii	19		21	+				0		d; e; k217
231	i	30			+		18		28		h9; l
246	iii	15	1	10	+		26	23	32	140	l
247	iii	15	10	15	+	*	26	52	30	248	l
249	iii	15	4	15	+	*			0		d
260	i	25	130		+	*	18	8	27	55	
261	i	19	85		+	*	18	6	30	55	
263	i	24	60		+	*	21	10	27	95	
264	ii	16	45	12	+	*	22		33		g16
266	iii	15	3	14	+	*	34	18	38	77	
267	vi	40							0		a; k205
268	v	19		14	+		26	5	45	63	
269	i	35				N	30	4	45	49	
271	i	24	45			*	25		36		i4
273	v	20	20	2	+	*	12		23		g19
274	iii	14	13	9	+		17	5	27	24	
276	v	16	4	14	+	*	20	7	32	58	
277	iii	14	1	10	+	*	23	7	37	56	
278	v	19	15	12	+		21		28		g13
279	i	30			+	*	26		64		g7; k261
280	v	18		18	+	*	31		37		g14

Table I (cont'd.)

1	2	3	4	5	6	7	8	9	10	11	12
281	v	12		24	+		36		49		g11
283	iii	17		11	+	*	25		36		g14
284	iv	14	1						0		b
285	iii	16		17	+	*	21		27		g9
286	iv	14	2		+		30		0		i1; k284
287	i	31	132		+	*	28		32		g17;j;k271
288	i	32				*	28		32		g8;j;k261
290	iii	21		7	+	*	27		31		g12
291	iv	23			+		21		26		i4
292	iii	15		16	+	*	22		29		i10;1
293	iii	15	6	18	+	*	29				i1;g4
294	v	21			+		17		24		i6;1
295	vi	22	18	1	+		13		20		g8; k294
298	vi	36	324	1°	contamination of all cultures						
299	vi	36	85	1°	contamination of all cultures						
300	iii	18	3	14	+		27		32		i4
302	iii	20	81	15	+	*	23		27		i6
304	v	34	300	13	+		16		25		i4; j
305	v	34	72	13	+	*	19		23		i5;j;k304
306	vi	29	150				18		25		i6
307	v	36	160				21		32		i4;j;k304
308	v	36	50			*	19		28		i6;j;k304
309	i	23		12	+	*	20		29		i4
321	iii	15		12	+		19		28		i5
322	iii	19		16	+	*	28		41		i7
323	iii	17		16	+		28		36		i5

Table I (cont'd.)

1	2	3	4	5	6	7	8	9	10	11	12
324	v	15		11	+		22		30		i8
325	iii	16	14	15	+	*	30		42		i6

Footnotes to Table I

71 - karyotype 47,XY,t(1;12)(p21;q21)

207 - karyotype 47,XXX

227 - karyotype 47,XX,+18

273 - fetus anencephalus

294 - fetus anencephalus

295 - fetus anencephalus

298 - fetus anencephalus

304 - fetus anencephalus

306 - fetus anencephalus

307 - fetus anencephalus

Key to Table I

Column numbers:

1. Laboratory number allotted to amniotic fluid.
2. Means of or reason for collection of amniotic fluid:
   - (i) Rhesus iso-immunised pregnancy.
   - (ii) hysterotomy specimen.
   - (iii) diagnostic amniocentesis for fetal chromosome studies.
   - (iv) diagnostic amniocentesis for possible inborn error of metabolism.
   - (v) diagnostic amniocentesis for α-fetoprotein estimation.

(vi) at rupture of membranes for the induction of labour.

3. Gestation of pregnancy from which amniotic fluid was collected (weeks).

4. Cell count of amniotic fluid (1000 cells/ml. of amniotic fluid).

5. Time required for a cytogenetic result when this was obtained in the minimum time (days).

6. Cytogenetic studies carried out but not necessarily on the primary culture or in the minimum time, +; cytogenetic failure, 0; blank space indicates cytogenetic studies not attempted.

7. Chromosomal sex of amniotic fluid: *, male; N, not known; blank space, female.

8. Time from setting up primary culture until subculturing it (days).

9. Number of passages for which cell strain derived from the amniotic fluid could be subcultured, using a 1:2 subcultivation ratio, before the cell strain died.

10. Time required to achieve a biochemical result (days); biochemical failure, 0.

11. Time as a cell strain (days).

12. Other comments:

(a) Primary culture never showed any signs of cellular proliferation.

(b) Primary culture degenerated and could not be subcultured.

(c) Primary culture lost to microbial contamination

before any assessment of growth or cytogenetic studies were possible.

(d) Primary culture lost to microbial contamination after cytogenetic studies had been completed.

(e) Karyotype other than normal, as stated in footnote.

(f) In vitro chromosome changes in cell strain.

(g) Mycoplasma contamination, culture discarded at ..... passage.

(h) Microbial contamination, culture discarded at ..... passage.

(i) Cell strain not cultured to senescence, serial cultivation terminated at ..... passage.

(j) Primary culture not set up until the day after the amniotic fluid was collected.

(k) Sample from the same pregnancy as (previous laboratory number).

(l) Cell strain tested for mycoplasma contamination and reported negative.

Table II. Data pertaining to all urines in this study which showed cellular proliferation in tissue culture.

1	2	3	4	5	6	7	8	9	10	11	12	13
52	i	1d	35		17	+	*	18	12	30	111	1
53	i	1d	32		12	+		15	18	23		f;g18
54	i	2d	39		17	+	*	19		27		i5; 1
66	i	1d	37			+		48		55		f; g35
68	i	1d	40			+	*	42	8	56	62	1
77	i	2d	37	4.5		+	*	35	8	46	70	
78	i	1d	34	130			*	31				h1
80	i	1d	38	1				31				h1
93	ii	1d	32		15	+	*	26	14	35	94	e; 1
100	i	1d	38	130	14	+		18	19	30	98	1
104	i	23d		1		+	*	33	18	42	90	1
116	iii	6d	38			+		53	14	63	60	e;f;1
125	ii	4d	37			+	*	33		42		g17
126	iii	8d										b; 1
128	iii	2y				+	*	21	12	33	65	
130	ii	35d				+		32		41		e;g15; k116
131	iii	1d	35			+	*	38	4	50	31	1
134	ii	S.B	36			+	*	50		64		i4
135	ii	1d	30		14	+		20		27		g8
137	iii	13d		16		+		30		34		g27; 1
144	iii	8d		1			*					b, 1
145	ii	2d	36	41								b
147	ii	7d	28	44		+		25	11	42	111	1
148	ii	5d	39									b; 1
150	ii	1d	32			+	*	31	3	52	34	1

Table II (cont'd.)

1	2	3	4	5	6	7	8	9	10	11	12	13
154	ii	1d	38				*					b
163	ii	1d	32			+		28		37		h14 1
164	ii	S.B	38	6.5		+		26		37		h12 1
165	ii	17d					*	35		52		h11 1
174	ii	1d	34					43		56		i8
184	ii	1d	39			+		35	14	60	104	1
212	ii	4d	37				*	40		48		h15; 1
222	ii	1d	34				*	21		31		h9; 1
265	i	6d	37	2		+	*	34	7	43	61	
272	ii	1d	33			+	*	19		25		i3
275	ii	1d	34					23		34		i6
282	ii	S.B	18					37		48		g8
296	ii	1d	36				*	24		35		i6
297	ii	1d	28					23		33		i5
303	ii	2d	40		16	+	*	27		34		i9
310	ii	1d	32		17	+	*	27		38		i4
312	i	1d	36		14	+		20		37		i8
317	i	1d	40		11	+		16		30		i8
318	i	7d	34					16		24		i6
319	i	1d	40		10	+		16		32		i5
320	i	7d	34					32		40		i4

Footnotes to Table II

93 - karyotype 47,XXY

116 - karyotype 47,XX,+18/48,XX,+18,+fra

Key to Table II

Column numbers:

1. Laboratory number allotted to urine.
2. Means of collection of urine sample:
   (i) Urine collecting bag.
   (ii) Aspiration from bladder at post-mortem.
   (iii) By suprapubic aspiration from a living child.
3. Age of child, d - days; y - years; S.B. - still-born.
4. Gestational age of child (weeks) if postnatal age is less than one week.
5. Cell count of urine (1000 cells/ml. of urine).
6. Time required for a cytogenetic result from the primary culture when this was obtained in the minimum time (days).
7. Cytogenetic studies carried out but not on the primary culture or in the minimum time, +; blank space indicates cytogenetic studies not attempted.
8. Sex of child from whom urine was collected: * - male; blank space - female.
9. Time from setting up primary culture until sub-culturing it (days).
10. Number of passages for which cell strain derived from the urine sample could be subcultured, using a 1:2 subcultivation ratio, before the cell strain died.
11. Time required to achieve a biochemical result (days); blank space - no biochemical result.

12. Time as a cell strain (days).

13. Other comments:

As for Table I. It should be noted that urines to which comments (a) or (c) would have applied were not included in Table II.

Table III. Data on amniotic fluid from pregnancies on which diagnostic antenatal cytogenetic studies were carried out.

1	2	3	4	5	6	7				8	9
						a	b	c	d		
115	40	15	(i)	19	b		17		17	2/126	(i)
200	41	35	(vi)	16	a		5		5	0/12	(i)
216	41	16	(i)	19	b	1	7		8	1/16	(i)
217	41	16	(i)	culture failed, repeated as No. 227							
220	23	14	(ii)	38	b		3		3	0/3	(i)
226	19	16	(iii)	14	b	1	10		11	0/19	(i)
227	41	19	(i)	20	c		1	8	9	2/22	(i)
246	39	15	(i)	10	b		15		15	0/39	(ii)
247	41	15	(iii)	15	a	1	7		8	2/30	(i)
249	45	16	(i)	15	a	2	16		18	0/68	(i)
266	19	15	(ii)	14	a	2	17		19	4/173	(i)
274	41	14	(i)	9	b	1	19		20	3/107	(i)
277	20	14	(ii)	10	a		12		12	1/47	(i)
283	31	17	(ii)	11	a	1	19		18	1/56	(i)
285	43	16	(i)	17	a		15		20	4/51	(i)
290	26	21	(ii)	7	a	2	16		18	1/27	(i)
292	29	15	(iv)	16	a	1	14		15	3/63	(i)
293	25	15	(v)	18	a		9		9	3/17	(ii)
300	41	18	(i)	14	b	1	12		13	2/32	
302	45	20	(i)	15	a	2	17	2	21	1/26	
321	34	15	(iii)	12	b	2	18		20	24/200	
322	40	19	(i)	16	a		14		14	1/40	
323	41	17	(i)	16	b	2	11		13	4/28	
325	35	16	(i)	15	a	4	16		20	4/200	

Table III (cont'd.)

1	2	3	4	5	6	7				8	9
						a	b	c	d		
328	40	16	(i)	15	a		17		17	8/100	
330	40	14	(i)	8	b	2	10		12	1/41	
332	44	19	(i)	13	b		10		10	0/150	
340	41	18	(i)	13	b	2	18		20	12/120	
341	43	18	(i)	12	a	1	19		20	23/200	
342	42	15	(i)	15	a		12		12	3/45	
343	41	14	(i)	12	a		10		10	4/30	
344	40	16	(i)	10	a	1	14		15	1/29	
345	39	14	(ii)	13	a		20		20	22/200	

Footnotes to Table III

115 - one cell (-G,+C)

227 - karyotype 47,XY,+18, one cell -D

249 - one cell -C, one cell -G

266 - one cell -C, one cell -F

283 - one cell (-G,-C)

290 - one cell -G, one cell -C

302 - one cell -C, one cell -E, one cell +F, one cell +C

321 - one cell -E, one cell (-F,-G)

323 - one cell -G, one cell -C

325 - one cell -E, one cell -16, one cell -G, one cell (-D, -C, +ace), one cell Dr.

330 - one cell -C, one cell (-G,-F,+C)

340 - one cell -C, one cell -E

341 - one cell -D

344 - one cell -G

Key to Table III

Column numbers:

1. Laboratory number.
2. Maternal age (years).
3. Gestation of pregnancy (weeks).
4. Reason for amniocentesis:
   - (i) Advanced maternal age.
   - (ii) Previous child with Down's syndrome.
   - (iii) Family history of Down's syndrome.
   - (iv) Parental chromosome translocation heterozygosity.
   - (v) Mother was carrier of an X-linked disease.
   - (vi) Other reasons.
5. Time required to achieve chromosome result (days).
6. Karyotype
   - (a) normal male karyotype
   - (b) normal female karyotype
   - (c) other (see footnote to Table).
7. Cytogenetic data:
   - (a) number of cells with 45 chromosomes
   - (b) number of cells with 46 chromosomes
   - (c) number of cells with 47 chromosomes
   - (d) total number of cells examined.
8. Ratio of polyploid cells/total number of cells examined for ploidy.
9. Outcome of pregnancy:
   - (i) As predicted.
   - (ii) As predicted, pregnancy terminated.

   Blank - pregnancy continuing.

Table IV. Data on amniotic fluids on which macrophage counts were performed.

1	2	3	4	5	6	7	8	9	10
273	iv	21	8	20	238		6226	2	iv
294	iv	17	8		254		1540		iv
295	v	18	20	18	250		1640	1	iv
298	v	36	10	324	6.4		660		iv
299	ii	36	25	85			4		iii
300	iii	18	15	3	10.6		10	14	i
302	iii	20	8	81	6.7		0	15	i
304	vi	33	18	300	26		590	13	iv
305	vi	33	12	71	1		6	13	i
306	v	29	20	150	44		630		iv
307	vi	35	10	160	21		10		iv
308	vi	35	10	50	3.5		10		i
309	i	23	5			0.16	102	12	ii
321	iii	15	18		7.7		6	12	i
322	iii	19	8		17.5		7	16	i
323	iii	17	6		12.2		2	16	i
324	iv	15	10		16.0		13	11	i
325	iii	16	7	14	19.2		12	15	i
326	ii	29	10	410	0.8		17		iii
327	iv	15	5		680		576		iv
328	iii	16	6		16.7		10	15	i
329	ii	21	8		5.0		4	15	i
330	iii	14	12	1	25.6		10	8	i
332	iii	19	2.5	37	31.1		20	13	i
333	iv	18	20		10.0		6	12	i
334	i	23	7	130		0.11	276		ii

Table IV (cont'd.)

1	2	3	4	5	6	7	8	9	10
335	i	32	7	125		0.02	43		ii
336	iv	16	10		13.4		20	18	i
337	ii	20	17	65	5.1		11	14	iii
339	iv	14	10	7	18.8		41	8	i
340	iii	18	10	76	2.8		10	13	i
341	iii	18	8	5	16.8		8	12	i
342	iii	15	4.5	30	13.4		25	15	i
343	iii	14	8	30	16.3		25	12	i
344	iii	16	8	8	16.3		20	10	i
345	iii	14	6	13	22.6		9	13	i
347	iii	16	12	30	13.8		8	10	i
348	iii	18	10	35	19.2		2	13	i
349	iii	16	8	16	17.3		16	12	i
351	ii	34	10	100	0.8		1		iii
353	i	33	5			0.04	1		ii
354	i	27	2.5			0.36	52		ii
355	i	32	8	330		0.05	28		ii
356	i	28	9	175		0.14	16		ii
357	vi	38	6	57			1		i
358	i	19	8	60		0.07	259		ii
359	i	35	7	10	4.0	0.05	14		ii
360	vi	38	4	57			14		vi
362	iv	18	7	52	6.0		445	16	vii
363	iv	15	10	1	25.4		3	14	i
364	iv	17	10	3	22.0		19	13	i
365	iv	17	12	13	7.7		1	13	i
366	iv	16	10	15	12.0		14	15	i

Table IV (cont'd.)

1	2	3	4	5	6	7	8	9	10
367	iii	14	8	3	181		168		v
368	iv	20	10	110	4.8		20	10	i
369	v	16	7.5	11	173		189		v
370	iii	13	5	1	143		82		v
371	iv	21	7	5	16.6		4		i
372	i	31	4			0.32	33		ii
373	iii	22	3.5	30	4.8		232	8	vii
374	iv	16	4	15	13.5		15	10	i
375	iv	18	10	35	13.5		40	10	i
376	iv	19	8		21.2		15		i
377	iii	16	5	4	23.4		2	14	i
378	iii	14	5	1	17.1		2	12	i
380	i	34	5	68		0.45	24		ii
381	v	15	1		118		84		v
382	iv	16	10	10	14.0		0	14	i
383	iii	16	1		20.2		16	13	i
384	iv	16	8	8	23.4		21	8	i
385	iv	18	5	18	12.7		12	12	i

Footnotes to Table IV

294, 295 - from the same pregnancy.
304, 305, 307, 308 - from twin pregnancy (see table 5.2).
333 - grossly bloodstained sample.
334, 335 - from the same pregnancy.
354, 372, 380 - from the same pregnancy.
367, 369 - from the same pregnancy.
370, 381 - from the same pregnancy.
376 - grossly bloodstained sample, failed to grow in culture.
381 - grossly bloodstained sample.

Key to Table IV

Column numbers:

1. Laboratory number allotted to amniotic fluid.
2. Reasons for collection of amniotic fluid:
   - (i) Rhesus iso-immunised pregnancy.
   - (ii) Hydramnios of unknown etiology.
   - (iii) Diagnostic antenatal chromosome studies.
   - (iv) α-fetoprotein estimation.
   - (v) At termination of pregnancy (including induction of labour at term).
   - (vi) Estimation of lecithin/sphingomyelin ratio.
3. Gestation (weeks) of pregnancy from which amniotic fluid was collected.
4. Volume (ml.) of amniotic fluid used to establish culture on which macrophage count was estimated.
5. Cell count (1000 cells/ml.) of amniotic fluid.
6. α-fetoprotein level (μg/ml) in amniotic fluid.
7. Optical density difference at 450 mμ of amniotic fluid.
8. Macrophage count (glass adherent cells/ 10 low power fields/ 10 ml. of amniotic fluid).
9. Time to cytogenetic result (days) when this was achieved in the minimum time.
10. Category of amniotic fluid (table 5.1 and Fig. 5.1)
    - (i) Normal.
    - (ii) Rhesus iso-immunised.
    - (iii) Hydramnios.
    - (iv) Anencephaly.
    - (v) Spina bifida.
    - (vi) Diabetic.
    - (vii) Other.

Appendix III

Publications

This appendix contains reprints of published works which form part of this thesis. The papers are presented in the order in which they were published. In the case of papers awaiting publication, photo copies of the proofs are included.

The following are the papers included in this appendix:

1. Sutherland, G.R. (1972). The role of amniocentesis in genetic counselling. Aust. J. Ment. Ret., 2, 85.
2. Sutherland, G.R., Bain, A.D. (1972). Culture of cells from the urine of newborn children. Nature, 239, 231.
3. Bain, A.D., Sutherland, G.R. (1973). Antenatal screening for Down's syndrome. Lancet, i, 423.
4. Butterworth, J., Sutherland, G.R., Broadhead, D.M., Bain, A.D. (1973a). Lysosomal enzymes of cultured amniotic fluid cells. Clin. Chim. Acta, 44, 295.
5. Sutherland, G.R., Grace, E., Bain, A.D. (1973a). Metaphase chromosomes from neonatal urine. Humangenetik, 17, 273.
6. Butterworth, J., Sutherland, G.R., Broadhead, D.M., Bain, A.D. (1973b). Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. I. α-glucosidase and β-glucosidase. Life Sciences, 13, 713.
7. Sutherland, G.R., Brock, D.J.H., Scrimgeour, J.B. (1973b). Amniotic fluid macrophages and

anencephaly. Lancet, ii, 1098.

8. Sutherland, G.R., Bain, A.D. (1973). Antenatal diagnosis of inborn errors of metabolism: Tissue culture aspects. Humangenetik, 20, 251.

9. Gordon, G., Sutherland, G.R., Bauld, R., Bain, A.D. (1974). The antenatal diagnosis of trisomy 18. Clin. Genet., 5, 110.

10. Sutherland, G.R., Bauld, R., Bain, A.D. (1974a). Observations on human amniotic fluid cell strains in serial culture. J. med. Genet. (in press).

11. Sutherland, G.R., Butterworth, J., Broadhead, D.M., Bain, A.D. (1974b). Lysosomal enzyme variations in thirteen cell strains cultured from one amniotic fluid. Clin. Chim. Acta, 52, 211.

12. Sutherland, G.R., Butterworth, J., Broadhead, D.M., Bain, A.D. (1974c). Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. II. α-galactosidase, ß-galactosidase and α-arabinosidase. Clin. Genet. (in press).

13. Butterworth, J., Sutherland, G.R., Broadhead, D.M., Bain, A.D. (1974a). Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. III. ß-glucuronidase, N-acetyl-ß-D-glucosaminidase, α-mannosidase and acid phosphatase. Clin. Genet. (in press).

14. Butterworth, J., Sutherland, G.R., Broadhead, D.M., Bain, A.D. (1974b). Effect of serum concentration, type of culture medium and pH on the lysosomal enzyme activity of cultured human amniotic fluid cells. Clin.Chim. Acta. (in press).

ust. J. Ment. Ret.
(1972) 2: 85-92

THE ROLE OF AMNIOCENTESIS IN GENETIC COUNSELLING

GRANT R. SUTHERLAND

Aust. J. Ment. Ret.
(1972) 2: 85-92

THE ROLE OF AMNIOCENTESIS IN GENETIC COUNSELLING

GRANT R. SUTHERLAND[1]

SUMMARY

The development of methods of diagnosing genetic disorders in the early fetus via amniocentesis are discussed. The role that this technique can play in genetic counselling is emphasised and it is stressed that this service should be offered to all couples who have a high risk of producing children with a disease that can be diagnosed *in utero*. It is also suggested that the screening of fetuses at moderate to low risk would be an economically viable public health measure which could significantly reduce the incidence of mongolism in the community.

One of the difficulties facing the genetic ɔunsellor is that he can usually only give ospective parents an indication of the risk volved in having an affected child. If this ;k is high then the parents may choose to ıve no children and request termination of ıy pregnancies which may arise. This can eate ethical problems which were summarised Pitt (1969) when he said "that to perform abortion for a one in four risk of abnor- ality is to run a three in four chance of losing normal infant". In cases where the risk is as gh as one in four, many parents would not be epared to take the chance of having, what ɔuld be to the majority in this situation, other affected child. When the risks are lower, y of the order of 5%, then decisions regarding rther children and those of terminating egnancy become more difficult. Such pro- ɛms could be largely overcome if any fetal normality could be accurately determined rly enough for therapeutic abortion of ected fetuses. This would allow parents th a high risk of having an affected child have a normal family and should go much the way to solving the problem outlined Pitt.

The problem of attempting to find out ıether a pregnancy will result in an affected ild has received increasing attention in the t few years. The main method of this fetal diagnosis is by a study of the amniotic fluid. In a procedure known as amniocentesis a sample of about 10 ml. of this fluid can be fairly readily obtained by puncture of the uterus from early in the second trimester onwards. The fluid so obtained is a clear amber colour and it contains a number of cells which are almost certainly of fetal origin (Votta et al., 1968) although their exact source is uncertain.

A full discussion of the techniques used to obtain amniotic fluid is beyond the scope of this paper. Early workers tried the transvaginal approach and had a high incidence of fetal complications. Riis and Fuchs (1966) had three spontaneous abortions and one stillbirth out of eleven cases using this method. The transabdominal approach (see Thiede, 1968 for details of this technique) seems to carry little risk. Gerbie et al. (1971) reported no complications, either fetal or maternal, after 256 such procedures and knew of 170 others which had been performed without complication. Ferguson-Smith et al. (1971) have had no complications in 30 cases and now use ultrasonography to locate the placental site and to exclude multiple pregnancy which is a contra-indication to amniocentesis.

A sample of amniotic fluid provides three avenues for study, the composition of the fluid itself, the cells present in the fluid and the culture of these cells.

Amniotic Fluid

There is controversy regarding the origin of amniotic fluid in early pregnancy (Emery,

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1970). However, there is little doubt that fetal urine contributes to the fluid and that this contribution becomes more significant as gestation proceeds. The total volume of amniotic fluid increases throughout pregnancy from 50 ml. at 12 weeks to 150 ml. at 15 weeks and 450 ml. at 20 weeks (Emery, 1970). As amniotic fluid is composed partly of fetal urine it would be reasonable to assume that it could be put through the series of tests which are normally applied to urine when screening for metabolic disorders. However, it will not be until normal values for all relevant components (as a function of gestational age) have been determined that the validity of this approach will be known. At present it is not known how the ability of the fetal kidney to secrete various metabolites varies with gestation and, possibly, with the diet of the mother. Nadler et al. (1970) reported a case of Pompe's disease in which the enzyme normally absent, α-1,4-glucosidase, was present in the amniotic fluid but not in the cells cultured from it; some enzymes in amniotic fluid may be maternal in origin. Matalon et al. (1970) reported that Hurler's syndrome could be diagnosed by the presence of excessive amounts of heparatin sulphate in the amniotic fluid at 14 weeks, Brock et al. (1971) failed to confirm this and missed diagnosing a case using similar criteria; genetic heterogeneity of this disease may explain this apparent contradiction. Jeffcoate et al. (1965) have diagnosed the adrenogenital syndrome by demonstrating grossly elevated levels of 17-ketosteroids and pregnanetriol in the amniotic fluid shortly before birth. As the source of these steroids is presumably the fetal urine and as the fetal adrenal cortex functions by the fourteenth week, the possibility of diagnosing this syndrome early enough for termination of the pregnancy arises. However, Merkatz et al. (1969) were unable to diagnose this syndrome in early pregnancy using these criteria, in fact, the steroid levels were only suggestive of the diagnosis at term.

To date, amniotic fluid studies have been generally equivocal and much more data on the composition of this fluid in normal and diseased states will be necessary before confident diagnosis of genetic disorders can be made early in pregnancy. When the production of an abnormal metabolite is not present at birth but depends on diet (e.g. phenylketonuria) it is difficult to see how the condition could be diagnosed from the amniotic fluid.

Amniotic Fluid Cell Studies

At present the information which can obtained from the cells without tissue cultu is limited to sex chromatin determination, t ABO blood groups and, recently, electr microscopy studies.

Sex Chromatin

In 1956, several workers independen found that fetal sex could accurately be dete mined by X-chromatin studies on the ce present in amniotic fluid. This technique h been extended, and probably increased reliability, since the discovery of the quinacri fluorescence pattern of the Y chromosome interphase nuclei (Pearson et al., 1970) w extended to amniotic fluid cells (Rook et a 1971). By combining these methods it shou be possible to accurately determine the s chromosome complement in all cases.

The determination of X-chromatin amniotic fluid cells was first used by Riis a Fuchs (1960) in the management of the pr nancies of two women who were carriers the gene for haemophilia. The technique however, applicable to all cases where an linked gene is responsible for a disease wh cannot be specifically diagnosed. When woman carries such a gene, half her s would be affected but all her daughters wou be normal. By terminating the pregnanc where the fetus is a male the parents concern can have a family of normal girls. This d not completely solve the problem of F (1969) although it reduces the chance losing a normal infant from three in four one in two and has been used by numer genetic counsellors to manage pregnanc involving X-linked conditions (e.g., Gerbie al. 1971) where male fetuses have been abo ed. Edwards (1970) has suggested that fe sex determination could be used as the basis aborting the female fetuses fathered by m who carry X-linked diseases (e.g. Haemophil as this " . . . could allow the reduction of s diseases to the level maintained by mutati the virtual elimination of familial cases, a progress in therapy without the prospect of creasing the incidence of disease."

Blood Groups

The determination of the fetal ABO bl groups is possible using cells from the amnic fluid as early as 11 weeks gestation (Sachs al., 1956). However, attempts to demonstr the Rh groups have been unsuccessful (S et al., 1969). Such information is of little

genetic counselling at present but may be ne useful when genetic maps of the auto- nes have been constructed, especially if it omes possible to determine other blood ups from these cells. In cases of close age where the probability of recombination small, it may be easier to determine the od group than to assay for its closely ed disease-producing gene. So far, the only close linkage known is that of the ABO is to the gene for nail-patella syndrome, and sibly the MNS blood group locus to that for rotylosis (Renwick, 1969).

ctron Microscopy Studies

The ultrastructure of normal amniotic fluid s has been studied by Hoyes (1968) who nd two recognisable cell types. Bartman 71) studied cultured amniotic fluid cells found only one cell type similar to fibro- ts cultured from other sources. Hug et al. 70) found that amniotic fluid cells obtained ing the 36th week of gestation from a man carrying a child subsequently shown to e Pompe's disease, had abnormal lysosomes suggested that it may be possible to diag- e this disease early in pregnancy by electron roscopy of uncultured amniotic fluid cells.

niotic Fluid Cell Culture Studies

This aspect of fetal diagnosis will probably ve to be by far the most important. Two ad fields of study are opened up by culturing cells present in the amniotic fluid, viz., genetics and biochemistry. Although much k has been done on the methodology of uring these cells, the optimum conditions e probably not yet been found. Hence, time required to produce results is longer n ideal especially when a result may indicate nination of a pregnancy; to produce a yotype requires two to three weeks and a chemical result may take significantly longer.

chemical Studies

Davidson (1970) lists about 30 different cell notypes which can be distinguished in ures of fibroblasts grown from skin biopsy. ris (1970) estimates that these represent ut one-third of the metabolic disorders in ch the biochemical defect is known. This wledge cannot be directly applied to fetal gnosis until it has been established that the s cultured from amniotic fluid cells similarly consistently express the same biochemical notypes as those grown from skin. It would necessary to establish this for each enzyme ther property separately and any variations during gestation would need to be known. For these reasons work on fetal diagnosis of biochemical disorders is proceeding slowly; each disorder requires a separate technique for diagnosis and each technique will require highly skilled staff, separate reagents and often expensive laboratory equipment. At present, biochemical techniques can only be applied to individual families who have already produced an affected child, the detection of heterozygotes in the population is not feasible unless the detection is reasonably simple and the gene frequency in the population is high, e.g., screening for carriers of Tay-Sachs disease in some Jewish populations where their frequency is as high as 1 in 30.

Biochemical studies on cultured cells involve three main types of approach, autoradiography, histochemistry and the assaying of enzyme levels on cell homogenates. These are all sophisticated methods which are prone to technical difficulties. The procedures are also lengthy, autoradiographs usually requiring at least a week to prepare after sufficient cells have been grown. However Fujimoto et al. (1968) reported the autoradiographic diagnosis of a female fetus heterozygous for the Lesch-Nyhan syndrome 22 days after amniocentesis. The main histochemical technique is the staining for metachromatic granules in the cytoplasm using toluidine blue. These granules have been demonstrated in a number of conditions including the polysaccharidoses and cystic fibrosis of the pancreas. It is, however, difficult to distinguish homozygotes from heterozygotes and the metachromasia may not develop until after two to three months in cell culture (Fratantoni et al., 1969), in fact this technique should not be used for diagnostic purposes at its present stage of development. The assaying of enzyme levels in cultured cell homogenates generally requires a large number of cells which may take several weeks to produce. However, specific enzyme deficiencies in cultured cells have been demonstrated for galactosemia, metachromatic leukodystrophy, lysosomal acid phosphatase deficiency and Pompe's disease (Nadler and Gerbie, 1971), in each case making intrauterine diagnosis possible. Regan et al., (1971) have diagnosed xeroderma pigmentosum, an autosomal recessive disease in which there is a lack of functional ultraviolet endonuclease, from cultured amniotic fluid cells. Their technique which was able to clearly distinguish normals from homozygotes and from heterozygotes was

based on shifts in the DNA sedimentation constants after the cells had repaired ultraviolet induced lesions in the presence of 5-bromodeoxyuridine and then been subjected to further irradiation.

Cytogenetic Studies

Shortly after Klinger first grew amniotic fluid cells in tissue culture, two small series of cultures reported by Steele and Breg (1966) and by Thiede et al. (1966) confirmed that the cells would grow in culture and that they could be used for chromosome analysis, thus making available a technique for the antenatal diagnosis of chromosome abnormalities. The bringing of this technique to perfection and its widespread application has been slow and is far from complete at present. It is, however, better developed than the majority of biochemical techniques, largely because the methods used are few, well established and largely within the competence of established cytogenetic units. Nevertheless, only one large series of cases of chromosome analysis as a result of amniocentesis has been reported, that of Gerbie et al. (1971) who successfully karyotyped 238 out of 250 samples of amniotic fluid. Such remarkable success has not been widespread, Nelson and Emery (1970) were successful in culturing only 49 out of 90 samples; although Ferguson-Smith et al. (197 were successful in 29 out of 30 cases referr for genetic counselling (repeat amniocente was, however, required in five cases) th managed to obtain karyotypes in only 17 24 samples obtained at hysterotomy.

The results of successful amniocentesis p formed for genetic counselling purposes wh required chromosome analysis are summaris in Table 1. The results reveal some surpris The finding of three pregnancies with Dow syndrome amongst 85 where the mother h had a previous regular mongol is, as Fergus Smith et al. (1971) point out, much high than the generally accepted recurrence risk this syndrome. Also the 5 abnormal fetu (three cases of Down's syndrome, one Klinefelter's syndrome and one XYY ma found in 130 women with advanced age at time of pregnancy (older than 40 years most cases) is more than expected. Advanc maternal age is a factor in the production both Down's and Klinefelter's syndromes the risk figures are generally believed to less than found here.

Whilst the theoretical expected risk of p ducing unbalanced offspring from Robertson translocations involving non-homologo chromosomes is 33%, it has been found practice to be in the region of 4-9% depend

TABLE 1

Cases in Which Fetal Chromosome Analysis via Amniocentesis has been Successfully used for Gen Counselling

Reason for amniocentesis	Valenti and Kehaty (1969)	Butler and Reiss (1970)	Gertner et al. (1970)	Nadler and Gerbie (1971)	Kersey et al. (1971)	Ferguson-Smith et al. (1971)	Total
Previous child with regular mongolism			5(1)	67(2)		13	85(3)
Previous child with other chromosome abnormality						1	1
Parent with balanced translocation	1(1)	2(1)		41(10)	1(1)	4	49(13
Maternal mosaic for mongolism						1	1
Increased maternal age	1		6(1)	119(4)		4	130(5)
Family history of mongolism			1			3	4
Exposure to mutagens	2		6			1	9
Total	4(1)	2(1)	18(2)	227(16)	1(1)	27	279(2

+ Three mongols, one XXY and one XYY male

Figures in parentheses indicate number of abnormal fetuses diagnosed and, except for two cases of Na and Gerbie, also indicate that these fetuses were therapeutically aborted.

the sex of the carrier parent and the exact romosomes involved (Hamerton, 1970). The asons for the finding of 13 out of 49 affected uses from balanced translocation carriers, parently in aggreement with the theoretical gure, is not known. Apart from the small mber of cases, a possible reason for the nding of an apparent excess of abnormal tuses in all the groups studied could be at these findings in mid-pregnancy should t be directly compared with expected fre-iencies of chromosome abnormalities in eborn neonates. With two exceptions, all e chromosomally abnormal fetuses were erapeutically aborted. It is possible that me of these would have spontaneously orted had they continued, this could have duced the incidence of neonates with romosome abnormalities amongst these oups closer to the expected levels. This is, wever, unlikely because most chromosomally normal abortuses are lost during the st trimester, and amongst mid-trimester ontaneous abortuses there are only about % with chromosome abnormalities (Carr, 70).

Hence it can be seen that antenatal chromome analysis is an effective means of precting the chromosomal status of the fetus and, followed by abortion of affected fetuses, can duce the incidence of chromosomal disorders birth. Unfortunately, misdiagnosis has curred and a normal fetus has been aborted at least one occasion (Kohn and Robinson, 70), however, no child with an unknown romosome abnormality has been born after nniocentesis although in the series of Gerbie al. (1971) two fetuses diagnosed as female amniocentesis were in fact males with rmal karyotypes. The reason for this error as probably an overgrowth of contaminant aternal cells.

ture Prospects

When any relatively safe technique which n prevent or improve the prognosis of a sease is discovered the question of screening pulations at risk arises. Amniocentesis is ch a technique, and it is worth considering nether it could be usefully employed as a reening test for disorders which can be diagsed *in utero,* either with a view to terminatg the affected pregnancies or initiating eatment of the fetus or the subsequent neote. The decision regarding termination or eatment or neither will depend on the particu- disease, the attitudes of parents, doctors and the law. In a liberal atmosphere fetuses with treatable disorders (e.g. phenylketonuria if it becomes detectable at this stage) would probably be terminated as would those with any sex chromosome abnormality and perhaps even those with balanced autosomal rearrangements, the termination of this last group is however difficult to justify on genetic grounds (Hamerton, 1970). In a more restrictive climate, probably only those with severely debilitating and untreatable disorders would be terminated.

Littlefield (1970) suggested that amniocentesis was appropriate for four distinct groups of women ranging from those with high risks of producing abnormal fetuses to those with a low to moderate risk. I would suggest that in the high risk cases amniocentesis is not only appropriate but its offer should be an essential part of genetic advice (where facilities are available) in the following instances:

1. Either parent carries a chromosomal rearrangement or has a chromosome abnormality, e.g. where the mother is XXX or the father XYY.
2. The parents are known to be carriers of autosomal recessive genes for any disease diagnosable *in utero.* These would normally have been ascertained via a previously affected child.
3. When one parent carries an autosomal dominant condition which can be diagnosed *in utero.*
4. When the mother is known to be the carrier of an X-linked condition, even when the specific condition cannot be diagnosed.

Where facilities are available amniocentesis is being offered in most of these cases at present and it can be expected that in the near future most major centres will have at least one unit capable of providing antenatal diagnostic services for such cases.

Apart from cytogenetic abnormalities, screening for other disorders is not feasible at present. This is because each requires a separate technique and even with considerable selection the incidence of fetuses with any single disorder is very low. The most common would probably be Tay Sachs disease with an incidence of about one per thousand in some Jewish populations but even here it is preferable to screen prospective parents for heterozygosity.

Apart from the high risk groups outlined above, there are two other groups which are probably worth screening for chromosome abnormalities. These are:

(a) Women who have produced a child with a chromosome abnormality.

(b) Women who become pregnant towards the end of their reproductive life when the chances of producing children with chromosome abnormalities is increased.

In general women in these categories are not being offered amniocentesis for several reasons. These include the inability of present cytogenetic units to handle the numbers which would be involved and reluctance on the part of obstetricians to perform a new procedure when its risks and benefits may not be fully understood and who are worried about the problem of technical failure to produce a result, necessitating repeat amniocentesis. The first problem can be overcome fairly easily because when the costs involved are compared to the resulting benefits, the saving to the community is great, hence government support for the establishment of adequate cytogenetic facilities should be forthcoming. The complications of amniocentesis, both fetal and maternal have been discussed and although present data suggests that this is a procedure which involves very low risks, more information is required from large series before this can be fully confirmed. The problem of failure will probably diminish as more experience is gained by laboratories in amniotic fluid cell culture.

If such a screening programme were applied to Australia, what could we expect? The mothers who had produced a previous child with a known chromosome abnormality would constitute only a small group and could probably be handled without expansion of existing facilities; this group will not be considered further. If 40 years was taken as an age above (and including) which women would be screened then in Australia in 1967 there would have been about 5,400 out of 230,000 mothers included (Sutherland, 1970). The chromosome abnormalities which this group would have been responsible for are estimated in table 2.

TABLE 2

Chromosome Abnormalities which would have been produced by 5,400 women 40 years or older in Australia in 1967

Abnormality		No. of Cases
Down's syndrome	(47,G+)	65
Edward's syndrome	(47,E+)	3
Patau's syndrome	(47,D+)	3
Klinefelter's syndrome	(47,XXY)	15
Triple-X female	(47.XXX)	5
"YY-males"	(47,XYY)	5
Other abnormalities		10

The cost to the community of these abno malities in human terms cannot be calculate it is sufficient to say that they will result considerable anguish to the families concerne It is, however, easier to calculate the cost financial terms, especially for the cases Down's syndrome which constitute the mo significant group. If each case required 2 years institutionalisation at a cost of $2000 p year, this amounts to $2,600,000. It is mo difficult to estimate the costs to the communi of the other abnormalities which would b found. The Edward's and Patau's syndrom cases could not be expected to live for mo than six months but would require hospit care for most of the time till death. It difficult to estimate the costs to the communi of the cases with sex chromosome abnormal ties. The XXY and XXX individuals do ha a tendency towards mental deficiency, b havioural disturbance and subsequent instit tionalisation (Bartholomew and Sutherlan 1971). They can develop normally except f sterility in the case of XXY, however th proportion of each which develops normally not accurately known. The XYY males prese even more of a problem, Australian experien (Sutherland et al., 1972) suggests that th have a tendency towards institutionalisatio but this is denied in Britain (Jacobs et a 1971). This is a problem which still requir much more study.

I would estimate that to screen these wome over 40 years would cost about $500,000 p annum (this allows for a centre in each state Hence the spending of about half a millio dollars would save about three millio Although these cost estimates are extreme approximate they are of the correct order magnitude, hence there would be about tenfold/fivefold return on money spent on th type of preventative medicine. The problem "discounting" is beyond the scope of this revie but it will tend to reduce the magnitude of th return on money spent on such populatio screening. However if the age above whic pregnant women were screened was lowered 35 years then it has been estimated (W.H.C 1972) that "the cost of such a programm would be substantially less than half that institutional care for the trisomic population If further experience with the technique amniocentesis confirms that the risks involve are almost negligible, or at least significant less than the chance of having a child with chromosome abnormality, then screening cou

extended to younger women, e.g. those in
35-39 age group. Carter (1970) stated
t in Britain, women of 35 and over produce
out half the mongols, yet comprise only
out 10% of the pregnant populuation.

I can only conclude by wholeheartedly
eeing with Ferguson-Smith et al. (1971)
en they say " . . . it is important that
niocentesis should not be undertaken . . .
trivial purposes such as choosing the sex
offspring".

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REFERENCES

tholomew, A. A., and Sutherland, G. R. (1971). sychopathy and sex chromosome abnormalities. aper presented at World Psychiatric Association onference, Mexico, November, 1971.

tman, J. (1971). Ultrastructure of cultivated nniotic fluid cells. *Obstet. Gynec.*, **38:** 838.

ck, D. J. H., Gordon, H. Seligman, H., and obo, E. de H. (1971). Antenatal detection of urler's syndrome. *Lancet*, (ii): 1324.

er, L. J., and Reiss, H. E. (1970). Antenatal tection of chromosome abnormalities. *J. Obstet. ynec. Br. Cwlth.*, **77:** 902.

, D. H. (1970). Chromosome abnormalities and ontaneous abortions. *Pfizer Medical Monoaphs*, **5:** 103. Edinburgh University Press.

er, C. O. (1970). Prospects in genetic counselg. In Emery, A. E. H., Editor, Modern Trends Human Genetics — **1**, London, Butterworths.

idson, R. G. (1970). Application of cell culture chniques to human genetics. In Emery, A. E. H., ditor, Modern Trends in Human Genetics — **1**, ondon, Butterworths.

ards, J. H. (1970). Uses of amniocentesis. *ncet*, (i): 608.

ry, A. E. H. (1970). Antenatal diagnosis of netic disease. In Emery, A. E. H., Editor, odern Trends in Human Genetics — **1**, London, tterworths.

uson-Smith, M. E., Ferguson-Smith, M. A., evin, N. C. and Stone, M. (1971). Chromosome alysis before birth and its value in genetic unselling. *Br. med. J.*, **4:** 69.

antoni, J. C., Neufeld, E. F., Uhlendorf, B. W., d Jacobson, C. B. (1969). Intrauterine diagsis of the Hurler and Hunter syndromes. *New gl. J. Med.*, **280:** 686.

noto, W. Y., Seegmiller, J. E., Uhlendorf, B. ., and Jacobson, C. B. (1968). Biochemical agnosis of an X-linked disease *in utero*. *Lancet*,): 511.

ie, A. B., Nadler, H. L., and Gerbie, M. V. 971). Amniocentesis in genetic counselling. fety and reliability in early pregnancy. *Amer. J. stet. Gynec.*, **109:** 765.

Gertner, M., Hou, L. Y. F., Martin, J., and Hirschhorn, K. (1970). The use of amniocentesis for prenatal genetic counselling. *Bull. N.Y. Acad. Med.*, **46:** 916.

Hamerton, J. L. (1970). Fetal sex. *Lancet*, (i): 516.

Harris, H. (1970). in Amniocentesis: *ad hoc* meeting of experts. Report of M.R.C. Conference, London, 1970.

Hoyes, A. D. (1968). Ultrastructure of the cells in the amniotic fluid. *J. Obstet. Gynec. Br. Cwlth.* **75:** 164.

Hug, G., Schubert, W., and Soukup, S. (1970). Prenatal diagnosis of type-II glycogenosis. *Lancet*, (i): 1002.

Jacobs, P. A., Price, W. H., Richmond, S., and Ratcliff, R. A. W. (1971). Chromosome surveys in penal institutions and approved schools. *J. med. Genet.*, **8:** 49.

Jeffcoate, T. N. A., Fliengner, J. R. H., Russell, S. H., Davis, J. C. and Wade, A. P. (1965). Diagnosis of the adrenogenital syndrome before birth. *Lancet*, (ii): 553

Kersey, J. H., Yunis, J. J., Lee, J. C., Bendel, R. P. (1971). Antenatal detection of partial C(6-12) trisomy. *Lancet*, (i): 702.

Klinger, H. (1965). Personal communication to Steele and Breg (1966).

Kohn, G., and Robinson, A. (1970). Tetraploidy in cells cultured from amniotic fluid. *Lancet*, (ii): 778.

Littlefield, J. W. (1970). The pregnancy at risk for a genetic disorder. *New Engl. J. Med.*, **282:** 627.

Matalon, R., Dorfman, A. Nadler, H. L., and Jacobson, C. (1970). A chemical method for the antenatal diagnosis of mucopolysaccharidoses. *Lancet*, (i): 83.

Merkatz, I. R., New, M. I., Peterson, R. E., and Seaman, M. P. (1969). Prenatal diagnosis of adrenogenital syndrome by amniocentesis. *J. Pediat.* **75:** 977.

Nadler, H. L., Bigley, R. H., and Hug, G. (1970). Prenatal detection of Pompe's disease. *Lancet*, (ii): 369.

Nadler, H. L., and Gerbie, A. (1971). Present status of amniocentesis in intrauterine diagnosis of genetic defects. *Obstet. Gynec.*, **38:** 789.

Nelson, M. M., and Emery, A. E. H. (1970). Amniotic fluid cells; Prenatal sex prediction and culture. *Br. Med. J.*, **1:** 523.

Pearson, P. L., Bobrow, M., and Vosa, C. G. (1970). Technique for indentifying Y chromosomes in human interphase nuclei. *Nature*, (Lond.), **226:** 78.

Pitt, D. (1969). The likelihood of congential disease. *Med. J. Aust.*, **2:** 712.

Regan, J. D., Setlow, R. B., Kabak, M. M., Howell, R. R., Klein, E., and Burgess, G. (1971). Xeroderma pigmentosum: A rapid sensitive method for prenatal diagnosis. *Science*, **174:** 147.

Renwick, J. H. (1969). Progress in mapping human autosomes. *Brit. Med. Bull.*, **25:** (i), 65.

Riis, P., and Fuchs, F. (1960). Antenatal determination of fetal sex in prevention of hereditary diseases. *Lancet*, (i): 383.

Riis, P., and Fuchs, F. (1966). Sex chromatin and antenatal sex diagnosis. In Moore, K. L., Editor, The Sex Chromatin. London and Philadelphia, Saunders.

Rook, A., Hsu, L. Y., Gertner, M., and Hirschhorn, K. (1971). Identification of Y and X chromosomes in amniotic fluid cells. *Nature* (Lond.), **230:** 53.

Sachs, L. Feldman, N., and Danon, M. (1956). Prenatal identification of blood group antigens. *Lancet,* (ii): 356.

Scott, J. S., Coulson, A., and Goulden, R. (1969). Antenatal determination of fetal blood groups. *J. Obstet. Gynec. Br. Cwlth.,* **76:** 330.

Steele, M. W., and Breg, W. R. (1966). Chromosome analysis of human amniotic fluid cells. *Lancet,* (i): 383.

Sutherland, G. R. (1970). Chromosome abnormalities in newborn babies. *Aust. J. Ment. Ret.,* **1:** 77.

Sutherland, G. R., Weiner, S., Bartholomew, A. A., and Fitzgerald, M. G. (1972). XYY males in Victoria. *Med. J. Aust.,* **1:** 1249.

Thiede, H. A. (1968). Obstetricians should le the technic of amniocentesis. *Obstet. Gynec.,* 146.

Thiede, H. A., Creasman, W. T., and Metcalf, (1966). Antenatal analysis of the human chron somes. *Amer. J. Obstet. Gynec.,* **94:** 589.

Valenti, C., and Kehaty, T. (1969). Culture of c obtained by amniocentesis. *J. Lab. clin. Med.,* 355.

Votta, R. A., de Gagneten, C. B., Parad, O., Giulietti, M. (1968). Cytologic study of amnio fluid in pregnancy. *Amer. J. Obstet. Gynec.,* **1** 571.

World Health Organisation Technical Report Se (1972), No. 497. Genetic Disorders: Preventi Treatment and Rehabilitation. World He Organisation, Geneva.

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Culture of Cells from the Urine of Newborn Children

THE increasing importance of amniocentesis for the diagnosis of disease *in utero* has focused attention on the cells obtained by this procedure. Culture of amniotic fluid cells is becoming an established procedure for the identification of foetuses with chromosomal and metabolic abnormalities. Cytogenetic and biochemical studies of these cells in culture and morphological studies of the uncultured cells have shown that they are of foetal origin[1–3]. However, the exact origin of the cells which grow remains speculative and several possibilities have been suggested including exfoliated cells from the amnion or from foetal skin[2,3] and also from the foetal respiratory[4] and urogenital[3,4] tracts. Since foetal urine undoubtedly contributes to the amniotic fluid as gestation progresses[5] it seemed reasonable to assume that some of the cells cultured from amniotic fluid could be derived from the foetal urinary tract.

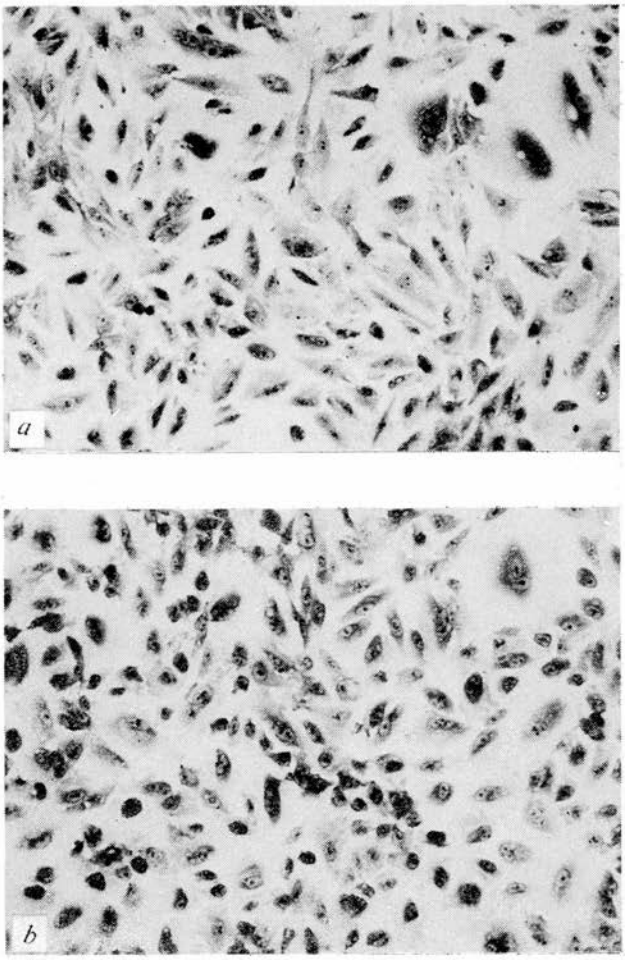

Fig. 1 Cells cultured from (*a*) urine and (*b*) amniotic fluid as seen 24 h after the third subculture (stain toluidine blue, × 100).

Samples of urine were collected in sterile bags from four infants less than two days old. For tissue culture, the urine samples were then treated as amniotic fluids; they were centrifuged at 1,000 r.p.m. for 10 min, the supernatant decanted, the cells resuspended in tissue culture medium and then transferred to Petri dishes containing cover slips. Ham's F10 TCM containing streptomycin and penicillin with the addition of

30% foetal calf serum was used. Cultures were incubated at 37° C in an atmosphere of 5% CO_2 in air. Although one culture showed contamination within 24 h, the remaining three were successfully maintained and proliferating cells were first observed at 6, 9 and 10 days respectively. Sufficient cells were present to allow harvesting for chromosome studies at 12 days in the first case and at 17 days for the other two. These culture times are similar to those being obtained at the present time in this laboratory for amniotic fluid cell cultures.

Using light microscopy the cultured urine cells appear morphologically similar to those cultured from amniotic fluid (Fig. 1); but further characterization of these two types of cell strain is required. Cell cultures have now been set up using urines from a total of eight infants aged less than 2 days and of gestational ages ranging from 31 to 40 weeks. Seven of these cultures have produced primary cell strains, one of which has now reached the seventh pass. A small number of cultures from older children have failed to grow. The only previous attempt to culture cells from urine is that of Jacobson and Barter[4] who set up cultures from sheep urines and bladder aspirates from four human foetuses; however, only one of the former was successful.

The culture of cells from neonatal urine, embodying simplicity in collection and in culture technique, could provide a non-traumatizing procedure for cytogenetic investigations when studies on tissue other than blood are required. Moreover, the most potentially useful application of this technique may be the production of primary cell strains for use in the investigation of inborn errors of metabolism at a biochemical and molecular level.

We thank Dr F. Cockburn for collecting the urine samples.

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[1] Van Leeuwen, L., Jacoby, H., and Charles, D., *Acta Cytol.*, **9**, 442 (1965).
[2] Votta, R. A., de Gagneten, C. B., Parada, O., and Giulietti, M., *Amer. J. Obstet. Gynec.*, **102**, 571 (1968).
[3] Wachtel, E., Gordon, H., and Olsen, E., *J. Obstet. Gynaec. Br. Commonw.*, **76**, 596 (1969).
[4] Jacobson, C. B., and Barter, R. H., *Amer. J. Obstet. Gynec.*, **99**, 796 (1967).
[5] Emery, A. E. H., in *Modern Trends in Human Genetics*—1 (edit. by Emery, A. E. H.), 268 (Butterworths, London, 1970).

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Letter to the Editor

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ANTENATAL SCREENING FOR DOWN'S SYNDROME

SIR,—Dr Stein and others (Feb. 10, p. 305) have analysed the feasibility of a screening programme for the prevention of Down's syndrome for New York City. They have shown that such a programme is desirable because of the increasing prevalence of Down's syndrome due to the increasing lifespan of affected individuals.

We agree with these authors that there is no justification in delaying the decision to undertake a diagnostic screening programme for Down's syndrome. If such a scheme can be shown to be feasible in New York City, we can see no reason why it should not be feasible in the United Kingdom and made readily available through the National Health Service.

The technique of amniotic-cell culture is well established, and for the antenatal diagnosis of Down's syndrome the success-rate is high and will probably be increased as more experience is gained. The nucleus of laboratory facilities and skill are generally available in the United Kingdom and phase I of the programme suggested by Stein et al. could probably be undertaken with only minimum expansion of existing services, since only about 2% of the pregnant population would be involved.[1] An additional benefit of such a scheme would be the prevention of other chromosome abnormalities, many of which also increase with maternal age and most of which have some associated morbidity.[2]

Whether we could agree with stages III and IV of the scheme proposed by Stein et al. is doubtful at present, as the cost of these stages would be high in relation to the benefits obtained, and the exposure of fetuses, which have only a very low risk of cytogenetic abnormality, to the small but finite hazards of amniocentesis may not be justified.

Only one small series of diagnostic amniocenteses in early pregnancy has been published in this country so far.[3] Does this reflect the general unpopularity of views such as those of Stein et al. and similar ones put forward by others ? [1]

Surely it is time for the evaluation in both economic terms and human considerations of a screening programme for the prevention of Down's syndrome in the United Kingdom. If such a programme was limited to stages I and II of Stein et al., it would pay for itself more than twice over,[4] and unless good reasons are found for not doing so, should be started forthwith.

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1. Amniocentesis: ad-hoc meeting of experts. Report of M.R.C. conference. London, 1970.
2. Sutherland, G. R. *Aust. J. ment. Ret.* 1972, **2**, 85.
3. Ferguson-Smith, M. E., Ferguson-Smith, M. A., Nevin, N. C., Stone, M. *Br. med. J.* 1971, iv, 69.
4. *Tech. rep. Ser. Wld Hlth Org.* 1972, no. 497.

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YSOSOMAL ENZYMES OF CULTURED AMNIOTIC FLUID CELLS

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:eceived October 25, 1972)

IMMARY

1. The levels of some lysosomal enzymes in a minimum of 38 amniotic fluid ll strains cultured to the third passage were as follows (mean ± standard deviation):

N-acetyl-β-D-glucosaminidase 52.1 ± 16.70, acid phosphatase 7.73 ± 2.82, β-lactosidase 4.59 ± 1.97, β-glucuronidase 1.30 ± 0.52, β-glucosidase 0.072 ± 0.030, galactosidase 0.42 ± 0.15, α-arabinosidase 0.53 ± 0.25, α-glucosidase 0.38 ± 0.18 d α-mannosidase 0.65 ± 0.30 nmole 4-methylumbelliferone/min/mg protein.

2. No correlation was observed between the levels of these enzymes and gesta-ınal age.

3. The levels of these enzymes in uncultured cells tended to be lower than in e corresponding cultured cells. Occasionally, very low levels of certain of the en-mes were found in uncultured cells, although normal levels were found in the rresponding cultured cells.

TRODUCTION

Amniocentesis combined with biochemical analysis of the amniotic fluid or cells increasingly being utilized for the antenatal diagnosis of genetic disease. The uses d possibilities of this procedure have been reviewed[1–3]. Great caution must be exer-ed if amniotic fluid or amniotic fluid cells are used directly for enzyme analysis[2]. A ·re reliable indication of the presence of enzyme activity in the foetus is obtained m cultured amniotic fluid cells[4]. However, the variation in the activity of lysosomal :ymes of cultured amniotic fluid cells has not previously been investigated in detail d is presented here.

TERIALS AND METHODS

Amniotic fluid samples were obtained from hysterotomy specimens and by niocentesis on rhesus isoimmunised women with no history of inborn errors of tabolism involving lysosomal enzymes. Primary cultures of cells from these fluids ·e established[5] and subsequently subcultured, using 0.25% trypsin in phosphate-fered saline to remove the cells from the Petri dish, into a glass culture bottle with

a growth surface area of approximately 20 cm². The cells were then similarly subcu tured into and maintained in bottles twice this size. Ham's F10 tissue culture mediu (Flow Lab. Ltd.) supplemented with 30% foetal calf serum (Bio-Cult Lab. Ltd.) wa used; antibiotics incorporated were kanamycin (100 μg/ml) or penicillin (60 μg/ml) an streptomycin (100 μg/ml) in combination. Culture bottles were flushed with 5% C in air prior to stoppering.

The cells were harvested on reaching confluency after the third subcultur Trypsin (0.25%) in phosphate-buffered saline was used to remove the cells from th glass. After washing in phosphate-buffered saline and centrifuging at 600 $\times g$ for 5 mi at 5° the cells were taken up in 4 ml of ice-cold 100 mM sodium chloride and sonicate for 15 sec at maximum power using a "Soniprobe" (Dawes Instruments). The hom genates were centrifuged at 25000 $\times g$ for 10 min at 5° and stored at −65°.

The methods of enzyme estimation were those of Butterworth *et al.*[6] with tw modifications:

β-glucuronidase—1 mM conjugate, 0.1 M acetate buffer pH 4.0, 30 min

α-glucosidase—2 mM conjugate, 0.2 M phosphate/citrate buffer pH 4.5, 60 mi

RESULTS

The results obtained for the enzymes assayed in a series of cultured amniot fluid cells are presented in Figs. 1 and 2. Table 1 gives the mean, standard deviati and range for each enzyme. The levels of the enzymes differed greatly, varying fro 52.1 ± 16.7 nmole/min/mg protein for *N*-acetyl-β-D-glucosaminidase to 0.072 0.030 nmole/min/mg protein for β-glucosidase. Each of the enzymes showed fairly narrow range of values except for a few cell strains showing unusually hi

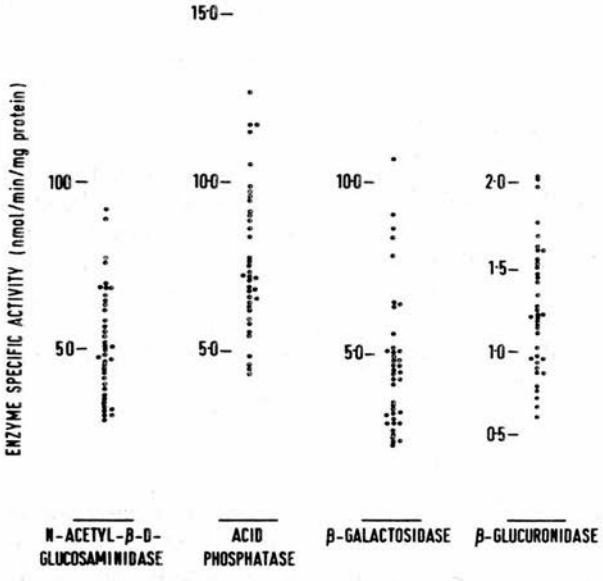
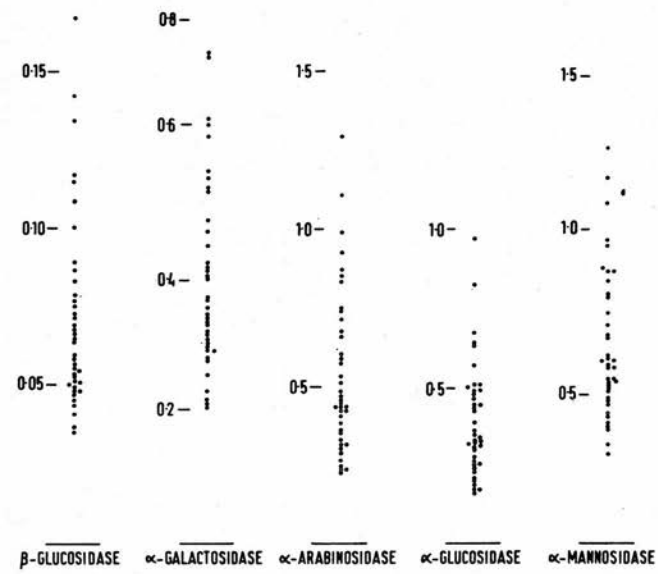


Fig. 1. Specific activities (nmole 4-methylumbelliferone/min/mg protein) of *N*-acetyl-β-D-glu saminidase, acid phosphatase, β-galactosidase, β-glucuronidase in amniotic fluid cells after third passage of culture.



. 2. Specific activities (nmole 4-methylumbelliferone/min/mg protein) of β-glucosidase, α-actosidase, α-arabinosidase, α-glucosidase and α-mannosidase in amniotic fluid cells after the rd passage of culture.

els of enzyme activity. Consequently 95% confidence limits based on a normal tribution produce lower limits for the enzyme specific activities which are well ow those actually measured. Hence Table I gives the upper and lower values nge) actually measured. The correlation coefficients (r) for the enzyme specific ivities with gestational age are given in Table I from which it can be seen that none the enzymes exhibits any relationship with this factor.

In Table II a comparison is made between the levels of the enzymes assayed uncultured and cultured amniotic fluid cells derived from three amniotic fluid nples. One feature, with five exceptions, is the lower level of these enzymes in the

BLE I

OSOMAL ENZYMES OF CULTURED AMNIOTIC FLUID CELLS

*yme	*Number of cell strains*	*Enzyme specific activity*[a]			*Correlation coefficient (r) of enzyme activity with gestational age*[b]
		Mean	*Standard Deviation*	*Range*	
\cetyl-β-D-lucosaminidase	39	52.10	16.70	28.70–91.85	−0.196
d phosphatase	38	7.73	2.82	4.34–12.69	0.198
alactosidase	41	4.59	1.97	2.37–10.69	−0.135
lucuronidase	38	1.30	0.52	0.62–2.02	0.284
lucosidase	39	0.072	0.030	0.03–0.17	−0.083
alactosidase	38	0.42	0.15	0.22–0.78	−0.158
rabinosidase	40	0.53	0.25	0.22–1.30	−0.081
lucosidase	40	0.38	0.18	0.17–0.97	0.218
annosidase	39	0.65	0.30	0.28–1.27	−0.093

nole 4-Methylumbelliferone/min/mg protein.
≥ 0.325 $P(r = 0) \leq 0.05$ df 35.

TABLE II

SPECIFIC ACTIVITY[a] OF LYSOSOMAL ENZYMES OF UNCULTURED AND CULTURED AMNIOTIC FLUID CEL

Enzyme	*Sample*[b]					
	Uncultured[1]	*Cultured*	*Uncultured*[2]	*Cultured*	*Uncultured*[3]	*Cultured*
N-Acetyl-β-D-glucosaminidase	8.28	52.37	23.18	47.26	4.46	31.40
Acid phosphatase	7.20	7.55	17.59	7.63	3.26	4.84
β-Galactosidase	0.10	4.36	0.30	3.91	0.25	4.19
β-Glucuronidase	2.20	0.94	2.94	1.34	0.73	1.02
β-Glucosidase	0.012	0.089	0.028	0.122	0.012	0.035
α-Galactosidase	0.023	0.37	0.075	0.35	0.018	0.32
α-Arabinosidase	0.008	0.43	0.040	0.43	0.040	0.60
α-Glucosidase	0.05	0.42	0.57	0.30	0.45	0.25
α-Mannosidase	0.25	0.55	0.28	0.62	0.20	0.50

[a] nmole 4-Methylumbelliferone/min/mg protein.
[b] Gestational age: Sample 1: 23 weeks; 2: 21 weeks; 3: 16 weeks.

uncultured compared with the cultured cells. The low level in uncultured cells of so of these enzymes, such as β-galactosidase, α-galactosidase and α-arabinosidase, wo make it very difficult to assert that an absence of enzyme in this material was d to a possible inborn error of metabolism. The need for great caution in the use uncultured amniotic fluid cells for antenatal diagnosis is clearly demonstrated sample 1. In this sample, the levels of α-glucosidase and α-arabinosidase were v low in the uncultured cells, but were normal in the corresponding cultured cells.

DISCUSSION

Nadler[7] suggested that the activity of glucose-6-phosphate dehydrogenase cultured amniotic fluid cells may show a correlation with gestational age and remar that there was no such correlation for acid phosphatase, β-glucuronidase and α-glu sidase. Indeed, in the present study, this latter group of enzymes, together with other lysosomal enzymes assayed, did not show a correlation with gestational a A similar lack of correlation for some lysosomal enzymes has been reported in un tured amniotic fluid cells[8].

The results presented here form part of an investigation of factors affect the levels of lysosomal enzyme activity in cultured amniotic fluid cells. The t passage was the earliest at which it was possible to obtain sufficient cells for enzyme assays and still maintain all of the cell strains. The enzyme levels in the t passage can, however, be taken as representative of at least the first five passag The use of cells from the third passage results in some delay after amniocentesis be the enzymes can be estimated. This delay varied from 4–10 weeks, although 7 of the cell strains could be assayed before 6 weeks. This time-lapse could be a prob in relation to antenatal diagnosis. However, even by using the first passage a m mum of only 2 weeks could be saved.

The relative merits of uncultured and cultured amniotic fluid cells for the chemical studies involved in antenatal diagnosis of inborn errors of metabolism not yet resolved. The assay of uncultured cells, if feasible, would avoid the un tainty of culture and permit early diagnosis[10]. Indeed Tay–Sachs' disease[11]

ompe's disease[12] have been diagnosed *in utero*. However, the reliability of using un-ultured cells for diagnosing these diseases has been seriously questioned[13–15]. Nadler nd Gerbie[2] have emphasised the need for great caution in the direct assay of uncul-ured amniotic fluid cells due to the possibility of maternal blood cell contamination, nzyme instability and inadequate numbers of viable cells. They suggested that the nzyme of interest should be related to another control enzyme in order to minimise ny such problems. However, our results indicate that this could be hazardous as it is ossible for uncultured cells to have some enzymes virtually absent and others nor-al, whilst the corresponding cultured cells have all their enzyme activities in the ntrol range. In contrast, no enzyme level in cultured cells from the whole series of ormal amniotic fluid samples was low enough to suggest that a particular enzyme as absent. However, the suggestion of Nadler and Gerbie[2] should be applied when ing cultured amniotic fluid cells for antenatal diagnosis of inborn errors of metabolism volving lysosomal enzymes in order to check that the culture and assay conditions ve not produced uniformly low enzyme levels.

KNOWLEDGEMENTS

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FERENCES

A. MILUNSKY, J. W. LITTLEFIELD, J. N. KANFER, E. H. KOLODNY, V. E. SHIH AND L. ATKINS, *New Engl. J. Med.*, 283 (1970) 1370, 1441, 1498.
H. L. NADLER AND A. B. GERBIE, *Amer. J. Obstet. Gynecol.*, 103 (1969) 710.
B. HULTBERG, P. A. ÖCKERMAN AND S. SJOBLAD, *Europ. Neurol.*, 7 (1972) 101.
P. F. BENSON, *Proc. Roy. Soc. Med. (London)*, 64 (1971) 1137.
G. R. SUTHERLAND AND A. D. BAIN, *Nature*, 239 (1972) 231.
J. BUTTERWORTH, F. SCOTT, W. M. MCCRAE AND A. D. BAIN, *Clin. Chim. Acta*, 40 (1972) 139.
H. L. NADLER, *Biochem. Genet.*, 2 (1968) 119.
W. R. DEN TANDT, *Clin. Chim. Acta*, 40 (1972) 199.
J. BUTTERWORTH, G. R. SUTHERLAND, D. M. BROADHEAD AND A. D. BAIN, (1972) unpublished observations.
R. G. SUTCLIFFE AND D. J. BROCK, *Clin. Chim. Acta*, 31 (1971) 363.
L. SCHNECK, C. VALENTI, D. AMSTERDAM, J. FRIEDLAND, M. ADACHI AND B. W. VOLK, *Lancet*, i (1970) 582.
H. L. NADLER AND A. M. MESSINA, *Lancet*, ii (1969) 1277.
R. G. DAVIDSON AND M. C. RATTAZZI, *Clin. Chem.*, 18 (1972) 179.
H. L. NADLER, *Pediatrics*, 49 (1972) 329.
R. NAVON AND B. PADEH, *Brit. Med. J.*, 4 (1971) 17.

Humangenetik 17, 273—275 (1973)

Metaphase Chromosomes from Neonatal Urine

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Summary. Tissue culture of the cells present in the urine of neonates has been demonstrated to be a suitable means of obtaining metaphase chromosomes whenever cytogenetic studies are required on cells other than blood lymphocytes. The advantages of this type of cell culture compared with the use of fibroblast culture from skin biopsy are discussed.

Zusammenfassung. Es konnte gezeigt werden, daß Gewebekulturen von Zellen aus dem Urin Neugeborener sich zur Gewinnung von Metaphasenchromosomen eignen, wenn cytogenetische Untersuchungen nicht allein an Blutlymphocyten durchgeführt werden sollen. Es werden die Vorteile diskutiert, die diese Art der Zellkultur gegenüber der Fibroblastenkultur aus Hautbiopsien bietet.

Mitotic metaphase preparations for chromosome analyses in the neonate are usually obtained from blood lymphocyte cultures but if further studies are indicated then fibroblasts cultured from a skin biopsy are most commonly used. Skin fibroblast culture has the disadvantage that results are not obtained for many weeks, skin biopsy is a traumatising procedure and is consequently not readily repeatable. It is now possible to culture cells from the urine of the neonate (Sutherland and Bain, 1972) and these can be used for cytogenetic studies.

Materials and Methods

Urine is collected into standard sterile urine collecting bags and transported to the laboratory. The urine is centrifuged at 1000 r.p.m. for 10 min and the supernatant removed. The pellet of cells is resuspended in tissue culture medium and pipetted into a 50 mm plastic (Nunclon) petri dish containing 4 or 5 glass coverslips (6×22 mm). Duplicate cultures are set up, the cells from approximately 5 ml of urine being used for each. The tissue culture medium is Ham's F10 with 30% fetal calf serum added. Antibiotics incorporated in the medium are kanamycin, or penicillin and streptomycin in combination. The cultures are incubated at 37°C in an atmosphere of 5% CO_2 in air and left relatively undisturbed for 1 week after which time half the medium is replaced thrice weekly. The cultures are examined regularly for signs of cell proliferation (almost always first seen on one of the coverslips) which are usually observed after a period of 7 to 14 days.

When an area of proliferating cells appear large enough for harvesting, the medium is fully changed and incubation continued for a further 16 hrs. The coverslip, on which the cells to be harvested are attached, is then removed to a separate petri dish or suitable tube and covered in fresh medium to which Colcemid at a final concentration of 1 μg/ml has been added. Incubation is continued for a further 5—6 hrs after which time the medium is aspirated from the tube and replaced by warm (37°C) 0.075 M KCl. Hypotonic treatment is terminated after 5 min by the addition of an equal volume of fixative (1 part acetic acid to 3 parts methanol)

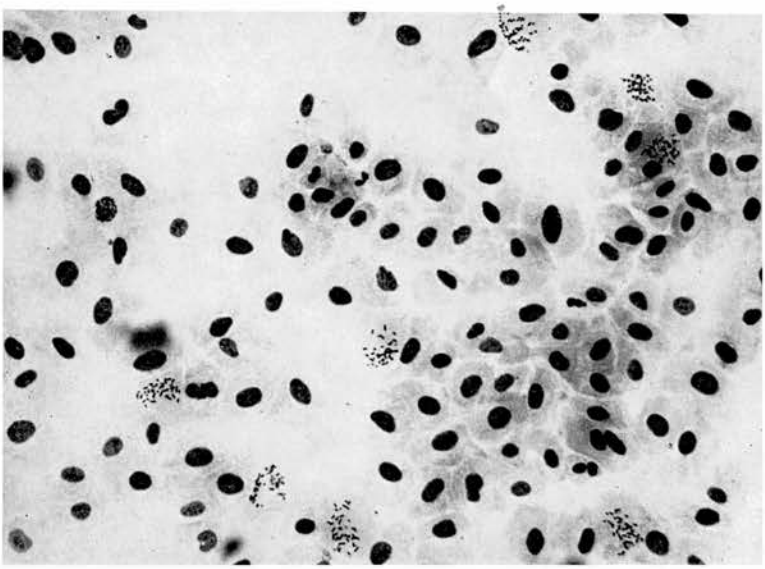

Fig. 1. Primary culture of urine cells harvested for cytogenetic studies after 12 days in culture and 5 hrs exposure to colcemid. (Stain Giemsa, ×125)

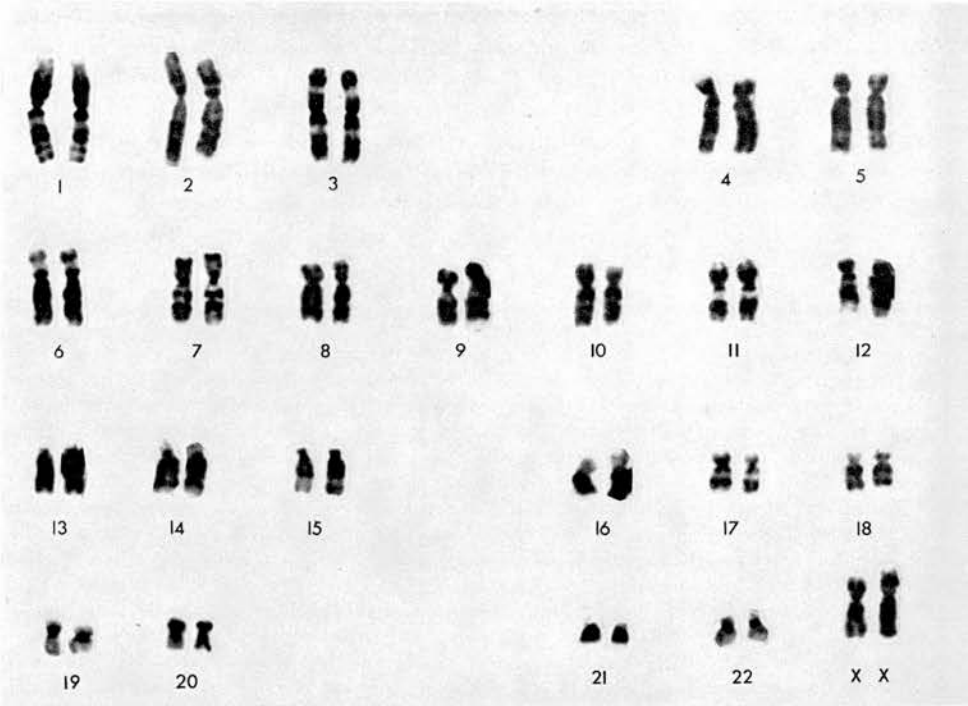

Fig. 2. Banded karyotype prepared using the method of Grace and Bain (1972) from a primary urine cell strain in the 13th passage

to the KCl. Half the KCl/fixative mixture is removed after 1 min and replaced by fixative. This step is repeated twice following which the coverslip is put through two changes of fixative, each of about 5 min. The coverslip is allowed to dry in air at room temperature, stained with Giemsa and mounted on a slide.

By allowing the cells remaining in the original petri dish to proliferate they can be subcultured to produce a primary cell strain suitable for further cytogenetic studies.

Results

A number of neonates have now been karyotyped successfully by tissue culture of urine. High mitotic activity is present in primary cultures harvested by this method (Fig. 1) and preparations suitable for fluorescent and banding studies have been obtained (Fig. 2).

Discussion

The technique of culturing urine cells for the purpose of cytogenetic study should prove a valuable addition to the techniques already in widespread use. It has several advantages over skin biopsy in that no trauma is involved in collecting the urine and repeat specimens can be readily obtained if contamination or other problems arise. The method is simple and should be within the capability of any laboratory involved in tissue culture. Results are obtained in 2—3 weeks, sooner than is usually possible in the case of skin biopsy and much less technical work is involved.

The main difficulty encountered is in the collection of the urine samples. Care should be taken as the urine may be contaminated by organisms which will multiply in the culture medium in spite of the antibiotics used. This problem could be overcome by collecting the urine via suprapubic bladder aspiration, a procedure certainly less traumatic than skin biopsy.

In the absence of microbial contamination the proportion of successful cultures has been high and would appear to be independent of the gestational age of the baby but the success rate almost certainly decreases as post-natal age increases. Possible factors which may influence the ability of the cells present in urine to proliferate in tissue culture are being investigated.

We are grateful to the staff of the Paediatric Special Care Unit of the Simpson Memorial Maternity Pavilion, especially Dr. F. Cockburn and Sisters B. Elliott and J. Shaw for collecting the urine specimens.

References

Grace, E., Bain, A. D.: A simple method for banded chromosomes. J. clin. Path. **25**, 910—911 (1972).

Sutherland, G. R., Bain, A. D.: Culture of cells from the urine of newborn children. Nature (Lond.) **239**, 231 (1972).

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LYSOSOMAL ENZYME LEVELS IN HUMAN AMNIOTIC FLUID CELLS IN TISSUE CULTURE. I. α-GLUCOSIDASE AND ß-GLUCOSIDASE

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SUMMARY

A number of factors which may correlate with the levels of α-glucosidase and ß-glucosidase in cultured amniotic fluid cells have been investigated. Fluctuations in enzyme activity occurred as passage numbers increased. Whereas α-glucosidase showed a consistently lower activity in the earlier passages compared to the later ones, the results for ß-glucosidase were equivocal. Both enzymes showed an increase in activity correlated with the time taken by the cells to reach confluency in the third passage. When replicate cultures were assayed daily after subculture, neither enzyme showed any change correlated with time. When cultures were grown in parallel in Ham's F10 and Eagle's M.E.M. tissue culture media, the activity of both enzymes was unaffected. Cell strains cultured from serial samples of amniotic fluid from the same woman had differing enzyme levels unrelated to gestational age.

A number of diseases have been shown to be due to a deficiency in the activity of a lysosomal enzyme (1,2,3,4). These enzymes are normally present in cultured amniotic fluid cells (5,6,7) and it has been found that an enzyme deficiency in the foetus is reflected in these cells (8,9,10). Although studies have been undertaken on the fluctuations in the activity of lysosomal enzymes in cultured fibroblasts (11-18), very little attention (11,19) has been paid to the variations in lysosomal enzymes in cultured amniotic fluid cells. With the rapidly expanding demand for antenatal diagnosis of inborn errors of lysosomal enzyme metabolism, a greater knowledge of the variations

in the levels of these enzymes in cultured amniotic fluid cells is necessary. Hence an investigation of some of the conditions of cell culture that may affect the levels of α-glucosidase and ß-glucosidase was undertaken.

METHODS

Amniotic fluid samples were obtained from hysterotomy specimens and by amniocentesis on Rhesus iso-immunised women who had no history of inborn errors of metabolism involving lysosomal enzymes. Primary cell cultures were set up (20) then subcultured, harvested, homogenized (7), and the lysosomal enzymes assayed using 4-methylumbelliferone conjugates (7,21).

The reproducibility of the combined culture and enzyme assay techniques was tested by establishing twelve replicate cultures of one cell strain at two different passages. The twelve replicates were harvested when they had simultaneously reached confluency and then assayed for enzyme activity. To study enzyme activity with respect to passage, six cell strains were subcultured for up to fifteen passages and the enzyme levels assayed at intervals. All the cell strains were assayed at the third passage and the time interval between subculture and harvest (confluency) noted. The variation in enzyme level within a passage was followed by establishing a series of replicate cultures which were harvested for enzyme assay at daily intervals for up to seven days. The effect of culture medium on enzyme activity was followed by performing enzyme assays on six cell strains cultured in parallel for three passages in Ham's F10 and Eagle's M.E.M. tissue culture media (supplemented with 30% foetal calf serum).

RESULTS

The reproducibility of the combined culture and assay system for each enzyme is presented in Table I. The variation of the total experimental procedure (coefficient of variance) was less than 10% for both enzymes. The variation in the levels of the enzymes with passage is given for six cell strains in Figs. 1 and 2. The coefficient of variance of the mean enzyme levels

TABLE I

Reproducibility of Culture and Assay System

Enzyme	Mean*	Standard Deviation	Coefficient of Variance (%)
α-GLUCOSIDASE	0.41[a]	0.035	8.5
	0.81[b]	0.027	3.3
ß-GLUCOSIDASE	0.154[a]	0.012	7.8
	0.053[b]	0.005	9.3

* Enzyme activity in nmoles 4-methylumbelliferone/ min./mg. protein for twelve replicates.
a - Assay tenth passage; b - Assay twentieth passage.

for the different cell strains was 8.9 - 42.6% for α-glucosidase and 32.9 - 65.0% for ß-glucosidase. These values are much greater than for the experimental procedure and indicate that there are changes occurring in the enzyme levels in any one cell strain as culture progresses.

Marked variations in the levels of both enzymes occurred with passage. In the case of α-glucosidase there was a rise in activity as culture progressed. This was shown by the lower activity ($P < 0.005$) of this enzyme in the first five passages

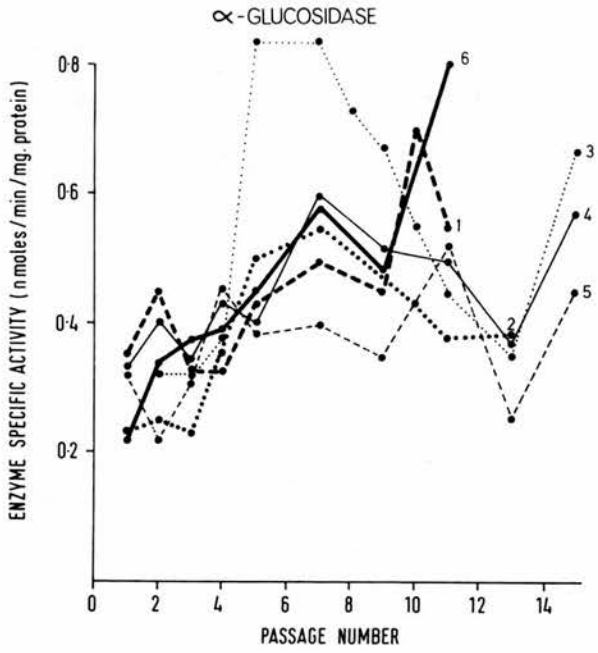

FIG. 1

The activity of α-glucosidase in serial passages of six strains of cultured amniotic fluid cells.

compared with the next ten. In addition the mean activity in the third passage was lower ($P < 0.01$) than that in the tenth passage of ten strains studied. For β-glucosidase cell strains 1 and 6 showed a positive correlation ($P < 0.05$) and strains 3,4 and 5 a similar negative one with passage number. Although the levels of this enzyme were also lower in the first five passages ($P < 0.05$) than in the next ten, the comparison of activities at the third and tenth passages showed no difference.

The relationship of the enzyme activity in cell strains at the third passage to the time at which confluency (harvest) was reached is given in Fig. 3. Both α-glucosidase ($r = 0.48$) and β-glucosidase ($r = 0.63$) showed a significant positive correlation with this factor. The correlation was still significant even if

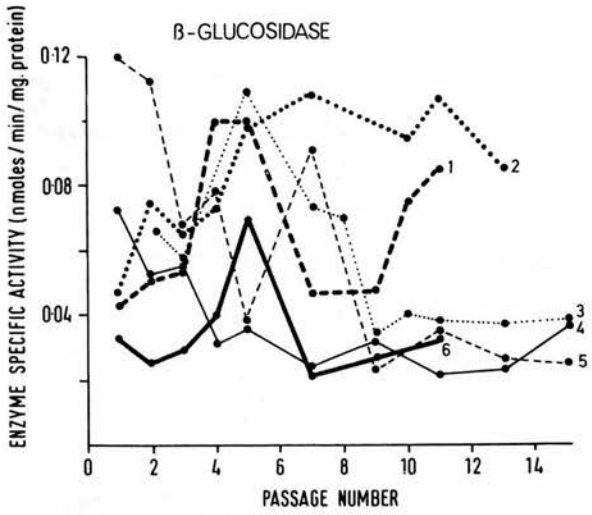

FIG. 2

The activity of ß-glucosidase in serial passages of six strains of cultured amniotic fluid cells.

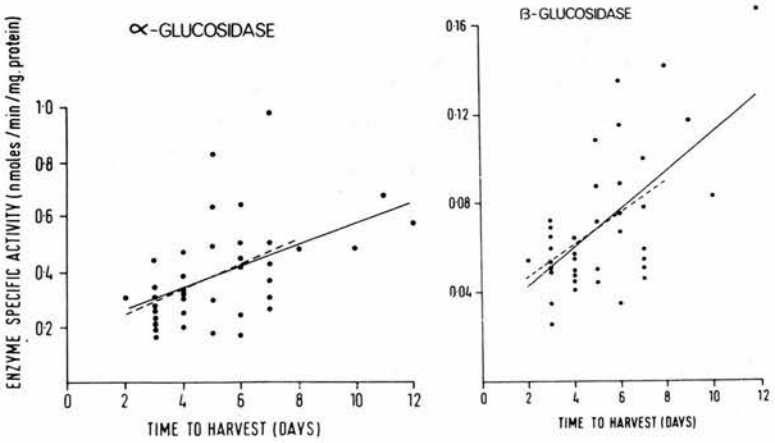

FIG. 3

Relationship of the activity of α-glucosidase and ß-glucosidase in cultured amniotic fluid cells with time to reach confluency (harvest).
——— All days ------ Without 9-12 days.

the few cell strains requiring 10-12 days to reach confluency were not considered.

The fluctuations in the levels of the two enzymes were not related to the time after subculture and no consistent change was associated with the point at which confluency was reached (Fig. 4).

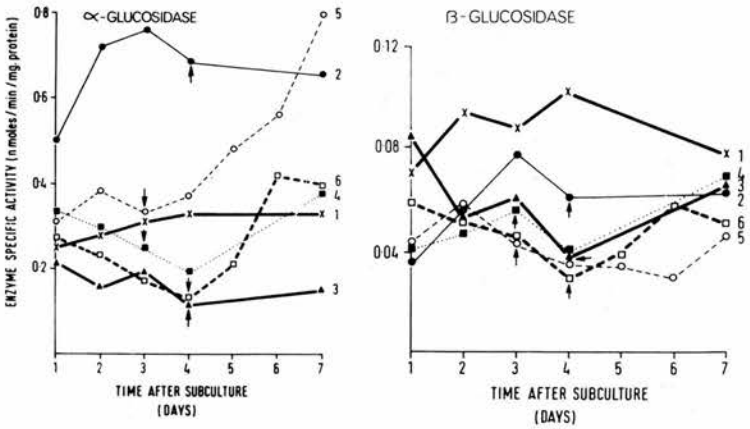

FIG. 4

The activity of α-glucosidase and ß-glucosidase in six strains of amniotic fluid cells in relation to time since subculture.

↑ Confluency; Strain 1 not confluent by 7 days.

Analysis of variance of the mean enzyme levels over three passages for each cell strain in the two culture media was performed. No significant difference was found for either α-glucosidase ($F = 2.1$, $F_{0.05}(1,5) = 6.61$) or ß-glucosidase ($F = 1.0$).

Cell strains were grown from serial samples of amniotic fluid obtained from several Rhesus iso-immunised women and the levels of the enzymes, at the third passage, are given in Table II. The activity of the two enzymes showed considerable variability within each series, but this did not seem to be related to the gestational age.

TABLE II

Enzyme Levels* in Cell Strains at the Third Passage Cultured from Serial Samples of Amniotic Fluid Obtained from Four Women

SERIES	GESTATIONAL AGE (WEEKS)	α-GLUCOSIDASE	ß-GLUCOSIDASE
1	18	0.30	0.059
	22	0.47	0.057
	24	0.33	0.048
	33	0.58	0.081
	35	0.24	0.025
2	23	0.38	0.064
	26	0.44	0.072
	28	0.20	0.069
3	22	0.57	0.167
	26	0.67	0.117
4	27	0.63	0.050
	34	0.31	0.049

* Enzyme activity - nmoles 4-Methylumbelliferone/ min./mg. protein.

DISCUSSION

Several studies have been undertaken of the influence of culture conditions on the levels of lysosomal enzymes in fibroblast-like cells (11-18). However, very little work of this nature has been reported using cultured amniotic fluid cells (11). This may well be due to the supposed difficulty in obtaining cell strains from amniotic fluid (22,23). However, in our laboratory it has been possible to culture the majority of the amniotic fluid cell strains to the tenth passage with some still growing well at the twentieth (24).

The fluctuations observed in the levels of the two enzymes

studied with passage has not been previously reported for amniotic fluid cells, although it has been noted for fibroblasts (25). The increase in the level of α-glucosidase with passage observed in this study probably reflects the decrease in the ratio of the epithelioid to fibroblast-like cells which occurs with time in culture (22). The situation with regard to ß-glucosidase is equivocal. Since the activity of ß-glucosidase, unlike α-glucosidase, can show opposite trends in different cell strains, it seems very unlikely that these trends can be related to changes in cell type. This interpretation is supported by the data of Gerbie et al. (19) on the activity of these two enzymes in epithelioid and fibroblast-like cultured amniotic fluid cells.

The positive correlation between the levels of both enzymes and time to harvest (confluency) is an unexpected result and may reflect the proportion of dividing to resting cells in cultures growing at different rates. The lack of any relationship between the enzyme activities and time after subculture is perhaps not surprising as such a relationship has only been demonstrated using homogeneous cell populations (13,16,17,18).

A significantly lower level of ß-glucosidase has been found in fibroblasts grown in Ham's F10 compared with Eagle's M.E.M. (11,17). Beutler et al. (11) claimed that this enzyme was also lower in activity in amniotic fluid cells grown in an enriched medium. In the present study, however, the high concentration of serum supplement used may have tended to obscure any real difference in enzyme activity attributable to the chemically defined components of the medium.

There has been no previous report on the levels of lysosomal enzymes in cells cultured from amniotic fluid obtained from the

same women at different stages of pregnancy. The apparent lack of a correlation of enzyme activity with the gestational age of the amniotic fluid agrees with results obtained using samples from different women (7).

The marked variations due to conditions of culture which occur in the levels of α-glucosidase and ß-glucosidase do not preclude the antenatal diagnosis of Pompe's or Gaucher's disease, but they would render the detection of heterozygotes very difficult. A complete understanding of the causes of these variations will require further investigation.

ACKNOWLEDGEMENTS

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REFERENCES

1. A. Milunsky, J.W. Littlefield, J.N. Kanfer, E.H. Kolodny, V.E. Shih and L. Atkins, New Engl. J. Med. 283 1370-1381, 1441-1447, 1498-1503 (1970).

2. H.L. Nadler, Birth Defects 6 26-33 (1970).

3. H.L. Nadler and A. Gerbie, Obstet. Gynec. 38 789-799 (1971).

4. B. Hultberg, P.A. Ockerman and S. Sjoblad, Euro. Neurol. 7 101-118 (1972).

5. M.M. Kabach and R.E. Cooke, Paediat. jap. 18 79-87 (1970).

6. P.F. Benson, Proc. R. Soc. Med. 64 1137-1139 (1971).

7. J. Butterworth, G.R. Sutherland, D.M. Broadhead and A.D. Bain, Clin. Chim. Acta 44, 295-299 (1973).

8. H.L. Nadler and A.M. Messina, Lancet 2 1277-1278 (1969).

9. B. Padeh and R. Navon, Israel J. Med. Sci. 1 259-263 (1971)

10. I.S. Salafsky and H.L. Nadler, J. Pediat. 79 794-798 (1971)

11. E. Beutler, W. Kuhl, F. Trinidad, R. Teplitz and H.L. Nadler, Am. J. hum. Genet. 23 62-66 (1971).

12. V.J. Cristofalo and D. Kritchevsky, Prog. Immunobiol. Stand. 3 99-105 (1969).

13. R. DeMars, Natn. Cancer Inst. Monogr. 13 181-195 (1964).

14. A. Maciera-Coelho, E. Garcia-Giralt, M. Adrian, Proc. Soc. exp. Biol. Med. 138 712-718 (1971).

15. S. Okada, M.L. Veath, J. Leroy and J.S. O'Brien, Am. J. hum. Genet. 23 55-61 (1971).

16. S.B. Russell, J.D. Russell and J.W. Littlefield, J. med. Genet. 8 441-443 (1971).

17. C.A. Ryan, S.Y. Lee and H.L. Nadler, Expl. Cell Res. 71 388-392 (1972).

18. J. Leroy and R. DeMars, Science 157 804-806 (1967).

19. A. Gerbie, S.B. Melancon, C. Ryan and H.L. Nadler, Am. J. Obstet. Gynec. 114 314-320 (1972).

20. G.R. Sutherland and A.D. Bain, Nature 239 231 (1972).

21. J. Butterworth, F. Scott, W.M. McCrae and A.D. Bain, Clin. Chim. Acta 40 139-142 (1972).

22. M.M. Nelson. In Antenatal Diagnosis of Genetic Disease (ed. A.E.H. Emery) Churchill Livingstone, London, 69-81 (1973).

23. J.W. Littlefield, Birth Defects 7 15-17 (1971).

24. G.R. Sutherland, R. Bauld and A.D. Bain. In preparation.

25. C.W. Castor, Arthritis Rheum. 14 41-54 (1971).

26. S.B. Melancon, S.Y. Lee and H.L. Nadler, Science 173 627-628 (1971).

Letter to the Editor

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AMNIOTIC-FLUID MACROPHAGES AND ANENCEPHALY

SIR,—Fetal macrophages have never been described in amniotic-fluid-cell culture. Elevated levels of α-fetoprotein in amniotic fluid are correlated with malformations of the fetal C.N.S., especially anencephaly.[10-15] The following two cases indicate that amniotic fluid from anencephalic pregnancies, diagnosed on raised α-fetoprotein levels, contains fetal macrophages.

Case 1.—This patient, aged 26, para 2+1, agreed to amniocentesis for amniotic-fluid α-fetoprotein estimation because her third pregnancy had resulted in an anencephalic stillbirth and her serum α-fetoprotein was raised.[16] At 21 weeks' gestation, amniocentesis produced about 20 ml. of slightly bloodstained amniotic fluid. The fluid was centrifuged and the cell pellet put into tissue-culture for chromosome studies.[17] The following day the cultures were noted to contain many macrophages, and a cytogenetic result was obtained within 48 hours of amniocentesis. Examination of 35 metaphases revealed only male cells. The α-fetoprotein level of the amniotic fluid was 238 μg. per ml. The pregnancy was terminated and resulted in an anencephalic male fetus.

Case 2.—This patient, aged 23, para 1+1, requested amniotic-fluid α-fetoprotein estimation because her first pregnancy had resulted in a spontaneous abortion at 10 weeks and her second in an anencephalic fetus at 36 weeks. Amniocentesis at 17 weeks' gestation produced 20 ml. of clear amniotic fluid. As in case 1, on the day following amniocentesis the culture was noted to contain considerable numbers of macrophages. The α-fetoprotein level of the amniotic fluid was 254 μg. per ml. Although ultrasonography on two separate occasions revealed no fetal malformation, the pregnancy was terminated by intra-amniotic prostaglandin and resulted in an anencephalic male fetus. Amniotic fluid obtained at termination had an α-fetoprotein level of 250 μg. per ml. The cells from this second sample of amniotic fluid were placed in tissue-culture, and again macrophages were present. A cytogenetic result was obtained within 24 hours; examination of 22 metaphases revealed only male cells. On the second day of culture the macrophages showed phagocytic activity towards sensitised sheep red cells; after 2 hours' incubation at 37°C about 70% of the macrophages had ingested red cells. Quinacrine-fluorescence studies of such macrophages indicated that about 80% of those which had ingested sheep red cells showed fluorescent Y-bodies.

These two cases provide evidence that fetal macrophages were present in amniotic fluid from anencephalic pregnancies. The fetal origin of the macrophages was demonstrated by their male karyotype. There was no evidence of

maternal macrophages, although in such circumstances a small number of these may not have been detected. The presence of maternal macrophages has been suggested as the explanation for the finding of female cells in amniotic fluid from male fetuses,[18] but this possibility has never been confirmed. The origin of the macrophages remains speculative, but they are most probably associated with the exposure of highly vascularised meningeal and cerebral tissue.

The findings reported here have important implications for the antenatal diagnosis of C.N.S. malformations. Any laboratory finding macrophages in amniotic-fluid cultures should investigate the possibility that the fluid may have come from a pregnancy in which the fetus has a gross C.N.S. malformation. Furthermore, if this finding of macrophages in anencephalic amniotic fluid is confirmed and extended to other C.N.S. malformations, then an additional indicator to α-fetoprotein levels will be available on which to base antenatal diagnoses. This may be especially helpful if α-fetoprotein levels are equivocal, or, as in the second case reported, ultrasonography fails to detect any malformation.

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10. Brock, D J. H., Sutcliffe, R. G. *Lancet*, 1972, ii, 197.
11. Brock, D. J. H., Scrimgeour, J. B. *ibid.* p. 1252.
12. Lorber, J., Stewart, C. R., Milford Ward, A. *ibid.* 1973, i, 1187.
13. Nevin, N. C., Nesbitt, S., Thompson, W. *ibid.* p. 1383.
14. Seller, M. J., Campbell, S., Coltart, T. M., Singer, J. D. *ibid.* 1973, ii, 73.
15. Allan, L. D., Ferguson-Smith, M. A., Donald, I., Sweet, E., Gibson, A. A. M. *ibid.* p. 522.
16. Brock, D. J. H., Bolton, A. E., Monaghan, J. M. *ibid.* p. 923.
17. Sutherland, G. R., Grace, E., Bain, A. D. *Humangenetik*, 1973, **17**, 273.
18. Uhlendorf, B. W. *in* Early Diagnosis of Human Genetic Defects (edited by M. Harris); Fogarty International Center Proceedings, no. 6. 1970.

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Antenatal Diagnosis of Inborn Errors of Metabolism: Tissue Culture Aspects

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Summary. The cells from 62 amniotic fluids have been cultured to the stage at which biochemical studies could have been undertaken. Although all cultures showed initial signs of cellular proliferation in only 90% of these were sufficient cells obtained for biochemical assay. If a time limit of 6 weeks was to be imposed, only 58% of the cultures could have been regarded as successful. The problems involved in culturing amniotic fluid cells for the antenatal diagnosis of inborn errors of metabolism are discussed.

Zusammenfassung. Von 62 Amnionflüssigkeits-Proben wurden die Zellen bis zu einem Stadium kultiviert, in dem biochemische Untersuchungen möglich wurden. Obwohl alle Kulturen anfänglich Zeichen einer Zellproliferation zeigten, wurden nur in 90% genügend Zellen für biochemische Untersuchungen gewonnen. Unter Annahme einer Zeitbegrenzung von 6 Wochen konnten sogar nur 58% aller Kulturen als erfolgreich betrachtet werden. Die Probleme bei der Kultivierung von Amnionzellen für die pränatale Diagnose angeborener Stoffwechselstörungen werden diskutiert.

Cultured amniotic fluid cells are almost always required when the antenatal diagnosis of an inborn error of metabolism is to be made. Except where histochemical techniques are available, this usually means many more cells are required than for cytogenetic studies. Gerbie *et al.* (1971) successfully karyotyped 95% of 250 amniotic fluids and other groups have reported similar high success rates. Little comparable data exists for success in culturing amniotic fluid cells to the stage where biochemical assays are possible. The time is usually limited to between 6 and 8 weeks for the culture of sufficient cells if a pregnancy may be terminated on the result. Hence it is valuable to report the experience in culturing cells from all the amniotic fluids received in this laboratory over a 1-year period.

Materials and Methods

Amniotic fluids were obtained from hysterotomy specimens and by amniocentesis of Rhesus iso-immunised women. The 62 amniotic fluids were taken from 47 women. When more than one sample was collected from any woman it was always from the same pregnancy. This study includes all such amniotic fluids received in this laboratory over the year ending November 1972.

The methods used for primary culture have been previously described (Sutherland *et al.*, 1973). The primary culture vessel was a 50 mm Nunclon plastic petri dish which contained four or five 6 × 22 mm glass coverslips. In the majority of cases one or two of these coverslips were removed from the culture for cytogenetic studies (Sutherland *et al.*, 1973). The primary

cultures were subcultured according to Butterworth *et al.* (1973) into a glass culture bottle with a growth surface area of 20 cm^2. On reaching confluency the cell strains were further subcultured into a glass culture bottle with a growth surface area of 40 cm^2. When the cell strains reached confluency after this second subculture the time since the amniotic fluid had been collected was noted and this was regarded as the time required to produce enough cells for a biochemical assay. Cell homogenates prepared from this quantity of cells contain ½—1 mg of protein and are adequate for a number of biochemical assays (Butterworth *et al.*, 1973).

Results

The results are summarized in Table 1 and Fig. 1. There were 62 amniotic fluids, 21 from hysterotomy specimens and 41 from Rhesus iso-immunised women. All cultures showed cell proliferation as observed under the inverted microscope.

Table 1. Data on the origin and cultural behaviour of the amniotic fluids in this series

Origin	No.	Gestational ages (weeks)		No. of biochemical failures	Time to biochemical result (days)	
		mean	range		mean	range
Rhesus	41	28.0	18—36	3	42.4	25—74
Hysterotomy	21	16.8	12—23	3	39.5	29—63
Total	62	24.2	12—36	6	41.4	25—74

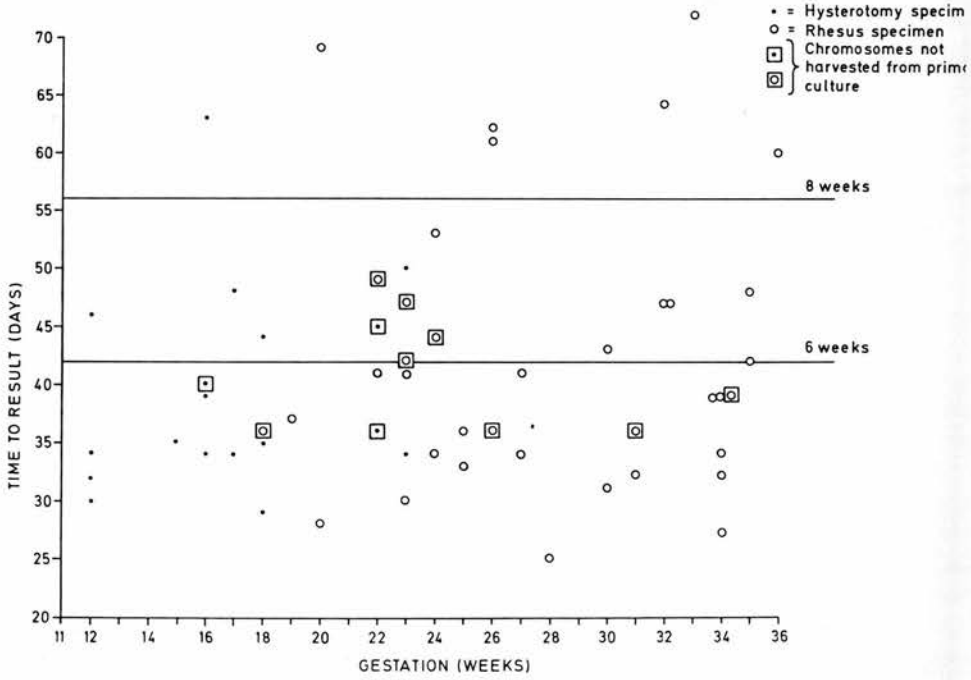

Fig. 1. Distribution of times taken to achieve sufficient cells for biochemical study

Table 2. Times required for biochemical and cytogenetic results from serial samples of amniotic fluid collected from three pregnancies

Pregnancy	Gestation	Time to biochemical result (days)	Time to cytogenetic result (days)
1	23	42	N
	26	36	N
	28	25	12
2	24	44	N
	30	43	18
	32	64	14
3	18	42	N
	22	41	26
	24	34	12
	33	74[a]	21
	35	42	19

[a] This cell strain degenerated after the third subculture.

N = Cytogenetic preparations not made in the minimum time.

There were two cytogenetic failures due to degeneration of the primary culture before a chromosome result could be obtained. Four other cultures were successful cytogenetically but failed from the biochemical point of view, one degenerated in primary culture after a chromosome result had been obtained, two were lost to microbial contamination after the first subculture and one degenerated after the first subculture. Hence, if there was no limit on the time taken to produce enough cells for biochemical assay, the success rate was 56 out of 62. If a time limit of 8 weeks was to be imposed then the success rate would fall to 49 out of 62 and if the time limit was to be reduced to 6 weeks then the success rate would fall to 36 out of 62 or 58%.

There is no correlation between gestational age and time to a biochemical result, either for the whole series or within the Rhesus or hysterotomy groups. Similarly, the mean time to such a result for specimens of less than 20 weeks gestation is not different from the time required by those of 20 weeks or more.

All except 11 primary cultures had at least one coverslip harvested for cytogenetic studies. It might be expected that removal of some of the earliest proliferating cells would increase the time required for biochemical success. This was not so as the time to result was not significantly different for the two groups.

The collection of serial samples of fluid from women with Rhesus iso-immunisation problems affords the opportunity of studying the behaviour of these serial samples in tissue culture. Three women contributed three or more samples and data on these are shown in Table 2. The times taken to reach a biochemical result within each series are no more related to each other than to the series as a whole. Although there appears to be some relationship between the times taken to achieve cytogenetic and biochemical results, a quick cytogenetic result does not necessarily mean that a quick biochemical result will follow.

Discussion

Commenting on tissue culture aspects of antenatal diagnosis, Brock (1973) has said "... that success to the biochemist is more elusive than success to the cytogeneticist". The main problem is one of time. Only 4 out of 62 amniotic fluids failed to reach a biochemical result because of their inability to proliferate sufficiently. Two were lost to microbial contamination; this should not happen and highlights the need for meticulous technique. In practice, if more than 6 to 8 weeks were required to produce enough cells any antenatal diagnosis for the purpose of terminating affected pregnancies would have to be regarded as a failure.

There is little data in the literature on this problem. Most authors discussing amniotic fluid cell culture have claimed the successful repeated subculture of primary cultures but the proportion in which this is achieved is usually not stated. The ability of these cells to proliferate sufficiently has been questioned by Littlefield (1971). Nadler and Gerbie (1970) were only able to subculture 75% of 155 successfully cultivated amniotic fluid cell strains more than three times. The most useful study in this regard is that reported by Uhlendorf (1970) who managed to achieve "massive cultures" from 77% of 114 amniotic fluid samples. 4—11 weeks were required to produce two 32 oz. prescription bottle monolayers, several subcultures being necessary. The time range was very similar to that in the present study although a higher number of cells were taken as the end point. In many cases "fairly large amounts" of amniotic fluid were used to initiate cultures whereas in the present series the volumes of fluid used were always less than 20 ml and often less than 10 ml.

The results from the serial samples taken during individual pregnancies indicate that each sample of fluid can behave differently. If as Uhlendorf (1970) has stated, there are less than 10 colony forming cells per millilitre of amniotic fluid, fluctuations in the number in any given sample of, say, 5 ml could be considerable. It is perhaps significant that although the cellular content of amniotic fluid increases throughout pregnancy (Nelson and Emery, 1970) the time required for a biochemical result is not related to gestation.

One problem not apparent in antenatal cytogenetic diagnosis is the degeneration of primary cultures. If an amniotic fluid culture for cytogenetic purposes is not proliferating after 7 to 10 days, amniocentesis can usually be repeated and another sample of fluid cultured. Most reported series of cytogenetic antenatal assessments contain a proportion of such instances. If, however, the primary culture grows well initially and then degenerates, it is usually too late to repeat amniocentesis and start again. A similar situation arises if degeneration takes place after the first or second subculture. A means of overcoming this problem, and of producing a quicker result in all cases, may be in the application of histochemical techniques. Uhlendorf (1970) described the use of histochemistry for the antenatal diagnosis of G_{M1}-gangliosidosis by staining cells for β-galactosidase activity. Hill and Puck (1973) have devised an autoradiographic method for the detection of galactosaemia which utilizes less than 1000 cultured cells. Such techniques could be carried out on primary cultures and perhaps produce a biochemical result in the same time as required for a cytogenetic result.

Nadler and Gerbie (1970) have stated "... the major limiting factor appears to be the difficulty in obtaining an adequate number of cells for biochemical analysis". Unless this problem can be overcome the chances of success in this type of antenatal diagnosis will remain low enough to give doubts as to its clinical application. Unless the techniques for the culture of amniotic fluid cells can be improved then efforts should be made towards developing histochemical techniques and micro assay methods for the enzymes involved in inborn errors of metabolism.

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References

Brock, D. J. H.: Biochemical studies on amniotic fluid cells. In: Emery, A. E. H., Ed., Antenatal diagnosis of genetic disease, pp. 82—112. Edinburgh-London: Churchill Livingstone 1973

Butterworth, J., Sutherland, G. R., Broadhead, D. M., Bain, A. D.: Lysosomal enzymes of cultured amniotic fluid cells. Clin. chim. Acta **44**, 295—299 (1973)

Gerbie, A. B., Nadler, H. L., Gerbie, M. V.: Amniocentesis in genetic counselling. Amer. J. Obstet. Gynec. **109**, 765—770 (1971)

Hill, H. Z., Puck, T. T.: Detection of inborn errors of metabolism: Galactosemia. Science **179**, 1136—1139 (1973)

Littlefield, J. W.: Problems in the use of cultured amniotic fluid cells for biochemical diagnoses. Birth Defects **VII**, 15—17 (1971)

Nadler, H. L., Gerbie, A. B.: Role of amniocentesis in the intrauterine detection of genetic disorders. New Engl. J. Med. **282**, 596—599 (1970)

Nelson, M. M., Emery, A. E. H.: Amniotic fluid cells; prenatal sex prediction and culture. Brit. med. J. **1970 I**, 523—526

Sutherland, G. R., Grace, E., Bain, A. D.: Metaphase chromosomes from neonatal urine. Humangenetik **17**, 273—275 (1973)

Uhlendorf, B. W.: Use of amniotic fluid and reliability of diagnostic procedures. In: Harris, M., Ed., Early diagnosis of human genetic defects. Fogarty International Proceedings No. 6, pp. 149—168 (1970)

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The antenatal diagnosis of trisomy 18

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The antenatal diagnosis of a fetus with trisomy 18 in a 41-year-old woman is reported. The pregnancy was terminated and the diagnosis confirmed cytogenetically and morphologically. The pathological findings in the fetus are discussed.

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The antenatal screening of mothers of advanced age for fetal chromosome abnormalities has been advocated by a number of authors. Although the main reason for this screening is the prevention of Down's syndrome (e.g., Stein et al. 1973), other chromosome abnormalities will inevitably be detected. We wish to report the antenatal diagnosis of a fetus with trisomy 18 in a 41-year-old mother and the subsequent pathological findings in the abortus.

Case Report

Pregnancy History

The pregnancy was the fifth pregnancy of a 41–year-old woman but the first with her second husband. In her first marriage two uncomplicated pregnancies were followed by two spontaneous abortions at 9 and 13 weeks.

She was first seen at 12 weeks of pregnancy when she expressed anxiety about the possibility of having a mongol child because of her age. In view of this anxiety amniocentesis was performed at 16 weeks' gestation when 8 ml of amniotic fluid was withdrawn. This fluid was unsatisfactory in culture (see below); consequently the amniocentesis was repeated at 19 weeks' gestation when 15 ml of fluid was withdrawn. At this time the uterus was found to be smaller than expected for the gestation. Negative Kleihauer counts were obtained after each amniocentesis and the fetal heart, monitored by Sonicaid, was unaffected. From this second sample of amniotic fluid the fetus was shown to have trisomy 18. Lower segment hysterotomy through a small Pfannenstiel incision was performed at 23 completed weeks of gestation and the conceptus removed intact. At this time the uterus was again noted to be small, as was the placenta. Post-operative recovery was uneventful. The mother is contemplating another pregnancy on the understanding that amniocentesis will again be performed.

Cytogenetic Findings

The first amniotic fluid sample contained very few cells and showed poor growth in culture. After 2 weeks there were only a few

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scattered proliferating cells, growing too slowly for chromosome studies. The cells obtained from the second sample did grow and after 20 days in culture a fetal karyotype of 47,XX,+18 was established.

The pregnancy was terminated by hysterotomy and cultures from fetal heart blood, skin and fascia all confirmed the antenatal diagnosis of trisomy 18 (see Table).

Pathological Findings

The fetus (Fig. 1) had a crown-rump length of 18.5 cm and a crown-heel length of 27 cm. There were multiple external abnormalities. The ears were low set with underdeveloped helices which were adherent to the scalp. There was a small preauricular skin tag on the right, and two subcutaneous nodules in the left preauricular area. There was micrognathia but no cleft lip and the palate appeared normal. Both hands showed flexion deformities of the fingers and the palmar creases were atypical. Both feet had rocker-bottom appearance and the first toes were shorter than the second. There was only a single umbilical artery.

Internally there also were multiple malformations. There was complete esophageal atresia with a tracheo-esophageal fistula. The heart showed a high interventricular septal defect. There was a complete horseshoe kidney. A Meckel's diverticulum was present with a small nodule of whitish tissue at its tip; histologically this proved to be pancreatic tissue without islet cells. The only abnormality of the brain was an anterior defect in the falx cerebri.

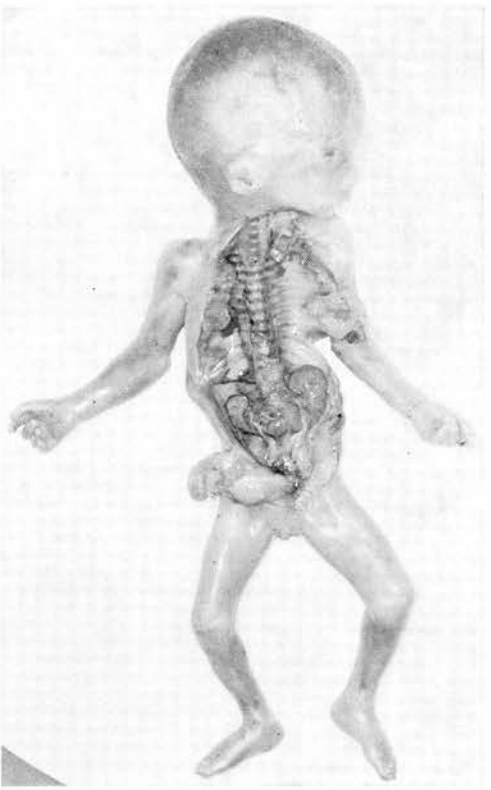

Fig. 1. The fetus at necropsy. Note low set ears, micrognathia, flexion deformities of the fingers and the horse-shoe kidney.

Table 1

Cytogenetic data

Tissue	Cells examined	Karyotype
Liquor amniotic fluid	9	47,XX,+18
Fetal heart blood	30	47,XX,+18
Fetal fibroblasts (a) skin	15	47,XX,+18
(b) fascia	15	47,XX,+18
Mother blood	30	46,XX
Father blood	30	46,XY

Discussion

The case illustrates two main points. The first is the value of antenatal diagnosis of fetal abnormality when this can be followed by abortion. The second is that chromosome abnormalities other than mongolism will be encountered when fetal karyotyping is performed on women of advanced age (Sutherland 1972). The prevention of children with these other chromosome abnormalities is an

added benefit in any attempt to reduce the incidence of Down's syndrome (Stein et al. 1973).

The amniocenteses were apparently without any ill effect. There were no maternal complications, the sac was intact at hysterotomy and the fetus showed no evidence of injury.

There are only two previously reported instances of the antenatal diagnosis of trisomy 18. The first, diagnosed at 31 weeks' gestation, proceeded to term and resulted in a stillbirth with the features of Edwards' syndrome (Milunsky et al. 1972). The second (Hsu et al. 1973) was a case similar to the present one, diagnosed in a 40-year-old woman and aborted at 20 weeks' gestation.

The pathological findings in the fetus are typical of those seen in neonates and older children with this syndrome (Taylor 1968). It is of interest that this fetus and the one described by Hsu et al. (1973) both had a Meckel's diverticulum with a small nodule at its tip. In the present case this proved to be heterotopic pancreatic tissue. One of the cases described by Taylor (1968) also had heterotopic pancreatic tissue but apparently not in association with a diverticulum.

The fetus had a crown-rump length which corresponded to only 20 weeks' gestation and the placenta was small. The finding of a small placenta in this syndrome has been recorded by Hecht (1963) and by Hsu et a (1973). The study of fetuses such as th may help to determine the time of onset c the retardation in growth and developmer usually associated with chromosome abnor mality.

References

Hecht, F. (1963). The placenta in trisomy 1 syndrome: Report of 2 cases. *Obstet. Gynec* **22**, 147–148.

Hsu, L. Y., L. Strauss, E. Dubin & K. Hirsch horn (1973). Prenatal diagnosis of trisomy 1 *Amer. J. Dis. Child.* **125**, 290–292.

Milunsky, A., L. Atkins & J. Littlefield (1972 Amniocentesis for prenatal genetic studie *Obstet. Gynec.* **40**, 104–108.

Stein, Z., M. Susser & A. Guterman (1973 Public screening programme for preventic of Down's syndrome. *Lancet* **i**, 305–310.

Sutherland, G. R. (1972). The role of amni centesis in genetic counselling. *Aust. J. mer Retardation* **2**, 85–92.

Taylor, A. (1968). Autosomal trisomy sy dromes: A detailed study of 27 cases Edward's syndrome and 27 cases of Patau syndrome. *J. med. Genet.* **5**, 227–252.

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Observations on human amniotic fluid cell strains in serial culture

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Summary. Observations made on 31 amniotic fluid cell strains serially cultured until senescent are recorded. The cell strains had an average life in culture of 13·9 passages (range 3–29). The source of the amniotic fluid from which the cultures were initiated did not influence the behaviour of the cell strains. The behaviour of the cell strains was unrelated to the growth characteristics of the primary cultures from which they were derived. Cell strains derived from serial samples of amniotic fluid from three women were compared and their characteristics were no more related to each other than to the group as a whole. The cell types found in amniotic fluid are described. The karyology of 12 of the cell strains was monitored and no significant changes from normal diploidy were seen. Possible reasons for the highly variable and unpredictable behaviour of amniotic fluid cell strains are discussed.

Cultured amniotic fluid cells are now in widespread use for the antenatal diagnosis of chromosome disorders and inborn errors of metabolism (Emery, 1970; Sutherland, 1972; Emery, 1973; Milunsky, 1973). An accurate knowledge of the behaviour of normal cultured amniotic fluid cell strains is therefore essential for their use in genetic studies. However, little appears to be known about the characteristics of these cells in tissue culture. It has been stated (Melancon, Lee, and Nadler, 1971) that there are at least two morphologically distinguishable cell types in amniotic fluid cell cultures—an epithelial type which cannot be subcultured more than two to five times and a fibroblast-like cell which can be cultured for more than 30 passages. On the other hand, Littlefield (1971) has said that '. . . 6 to 8 weeks of rapid culture can approach the limit of the growth potential of amniotic fluid cells . . .'. This report records observations made during the long-term culture of 31 amniotic fluid cell strains.

Materials and Methods

Amniotic fluids were obtained from hysterotomy specimens (HS), by amniocentesis on Rhesus isoimmunized women (RhS) and by artificial rupture of membranes (ARM) for the induction of labour. The primary cultures were set up as previously described (Sutherland and Bain, 1972) and subcultured according to the method of Butterworth *et al* (1973). The cell strains were maintained in 250-ml glass vessels with a growth surface area of 40 cm^2 and regularly subcultured on reaching confluency, using a 1:2 subculture ratio. Throughout this study, all cell strains were cultured in Ham's F10 tissue culture medium supplemented with 30% fetal calf serum and with the antibiotics kanamycin, or penicillin and streptomycin in combination. The methods used for cytogenetic studies on primary cultures have been described (Sutherland, Grace, and Bain, 1973b). Once cell strains became established, chromosome preparations were made using standard methods. Cell strains were stored in 10% dimethyl sulphoxide in culture medium in the vapour phase of a liquid nitrogen unit.

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Results

The 31 cell strains were derived from amniotic fluid samples taken from 25 women, ie, one woman contributed four samples, another three, and another two at different stages of pregnancy. The gestational ages, means of collection, and chromosomal sexes of the amniotic fluids are shown in Table I. All had normal karyotypes with the exception of two samples from one pregnancy which had a karyotype 46,XY,t(1;12)(p21;q21).

TABLE I

GESTATIONAL AGES, ORIGINS AND CHROMOSOMAL SEX OF THE 31 AMNIOTIC FLUIDS WHICH YIELDED CELL STRAINS

Origin of Amniotic Fluid	Gestation (weeks)	Chromosomal Sex	
		Male	Female
HS ($n=8$)		2	6
Mean	16·5		
Range	11–23		
ARM ($n=1$)	39	1	
RhS ($n=22$)		9	13
Mean	28·3		
Range	18–35		
Total ($n=31$)		12	19
Mean	25·6		
Range	11–39		

TABLE II

DATA ON 31 AMNIOTIC FLUID CELL STRAINS

Origin of Amniotic Fluid	No. of Passages	Days in Culture	Days as Cell Strain	Mean Interval between Subculture
HS ($n=8$)				
Mean	12·0	27·37	76·63	6·38
Range	4–20	18–29	19–135	4·8–9·6
ARM ($n=1$)	11	27	111	10·1
RhS ($n=22$)				
Mean	14·77	25·91	96·63	7·36
Range	3–29	19–41	31–146	3·0–12·0
Total ($n=31$)				
Mean	13·90	26·32	91·94	7·19
Range	3–29	18–41	19–146	3·0–12·0

Some data on the cell strains are shown in Table II. The differences between the HS and RhS are not significant for any of these parameters. The number of passages is the number of times a cell strain could be subcultured, using a 1:2 subcultivation ratio, before the cell strain died. Death of a cell strain was defined as the point at which it would no longer grow to confluency after subculture. The time as a cell strain was the interval between subculturing the primary culture and the death of the cell strain. The mean interval between subcultures was the time as a cell strain divided by the number of passages till death, and was a crude estimator of the overall rate of growth of a cell strain. The distribution of the number of passages is shown in Table III.

The data were examined to see if any of the parameters of the amniotic fluid were related to the behaviour of the cell strain in culture. There was no correlation between gestational age and the following: time in primary culture, the number of passages

TABLE III

THE DISTRIBUTION OF THE NUMBER OF PASSAGES FOR WHICH THE 31 AMNIOTIC FLUID CELL STRAINS COULD BE CULTURED UNTIL DEATH

No. of passages	0–4	5–8	9–12	13–16	17–20	21–24	>25
No. of cell strains	3	3	8	7	5	3	2

to which a cell strain could be cultured or the mean interval between subcultures. Similarly, the number of passages was not correlated with the time in primary cu lture. There was, however, a significant ($p<0·01$) correlation ($r=-0·550$) between mean subculture interval and the number of passages (Fig. 1).

All cell strains which survived more than five or six passages were stored in the liquid nitrogen unit. Thirteen strains have been retrieved and then cultured to senescence. The mean number of passages before death of the 13 strains was 14·5 (range 5–25) and after storage the mean was 13·4 (range 5–24). Most of the retrieved cell strains died within ± 3 passages of the corresponding strain which had not been stored. The greatest difference was one strain which survived for 17 passages before storage and for only 12 passages after being stored at the fourth passage.

When more than one amniotic fluid sample was collected at different stages from a single pregnancy it was possible to compare the cell strains derived from these samples. Some data relating to three such groups of cell strains are shown in Table IV.

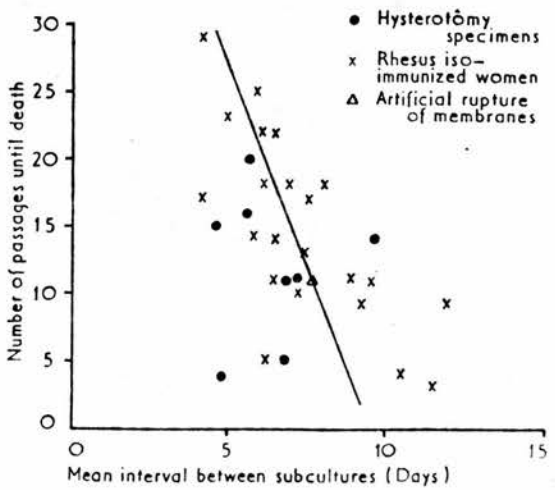

FIG. 1. Relationship between the number of passages for which cell strains could be cultured before death and mean interval between passages. ($r=-0·550$.)

TABLE IV

DATA ON 9 AMNIOTIC FLUID CELL STRAINS COLLECTED FROM THREE PREGNANCIES

Group	Chromosomal Sex	Gestation (weeks)	Days in 1° Culture	No. of Passages	Days as Cell Strain	Mean Interval between Subcultures
1*	Male	22	28	11	106	9·7
		26	41	4	42	10·5
2	Female	18	28	18	144	7·8
		22	24	8	108	12·0
		24	20	15	110	7·3
		35	36	17	69	4·1
3	Female	23	26	18	125	6·9
		26	26	22	129	5·9
		28	19	10	71	7·1

* This group has a constitutional chromosome abnormality (see text).

The cell strains in each group do not show uniform behaviour, they vary as greatly as cell strains derived from different pregnancies. This may indicate that the genetic make-up of the cells is not responsible for the variable behaviour of the cell strains.

The cell morphology was monitored throughout the period of culture by direct observation under the inverted microscope and by the examination of Giemsa-stained monolayers grown on flying coverslips. There were five readily recognizable cell types.

1. Macrophages (Fig. 2). These were visible the day after primary cultures had been set up. These appear to divide in culture but cannot be subcultured. They degenerate and are overgrown by other cell types. Very few macrophages are seen in amniotic fluid cell culture except when the amniotic fluid comes from an anencephalic pregnancy when they are present in considerable numbers (Sutherland, Brock, and Scrimgeour, 1973a).

FIG. 2. Macrophages from anencephalic amniotic fluid after 20 hours in culture. (Giemsa stain. × 200.)

2. Epithelioid Type I Cells (Fig. 3) which grow in 'islands' in primary culture. These cells can apparently be subcultured but lose their tendency to grow in 'islands' after subculture.

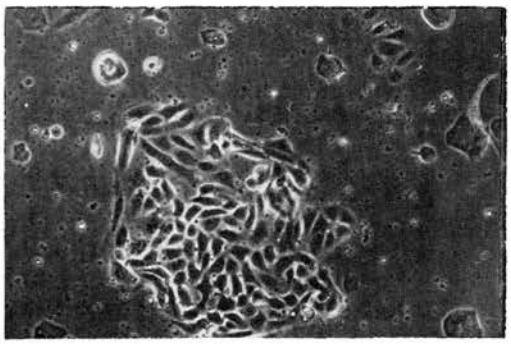

FIG. 3. Epithelioid type I cells after 18 days in primary culture. (Phase contrast, × 200.)

FIG. 4. Epithelioid type II cells after 23 days in primary culture. There are fibroblast-like cells growing between the epithelioid cells. Note one binucleate epithelioid cell and the fibrillar nature of the cytoplasm. (Phase contrast, × 200.)

3. Epithelioid Type II Cells (Fig. 4). These are very large cells with much cytoplasm and sometimes with more than one nucleus. Under phase contrast the cytoplasm of these cells has a fibrillar appearance. These cells can be subcultured at least 20 times. Almost all primary cultures of amniotic fluid contain these cells.

4. Epithelioid Type III Cells (Fig. 5) which morphologically resemble type I except that they do not grow in islands in primary cultures. In their growth patterns these cells show some of the features of the next cell type. These cells can be readily subcultured.

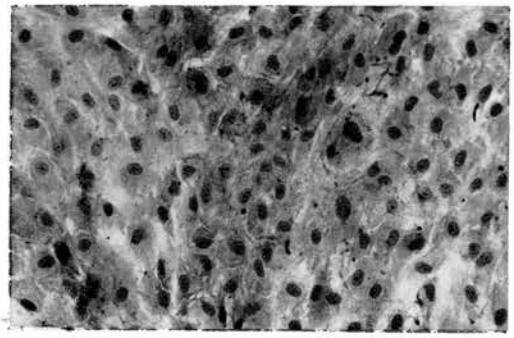

FIG. 5. Epithelioid type III cells in the twenty second passage of culture. (Giemsa stain, × 200.)

5. Fibroblast-like Cells (Fig. 6) which can be readily subcultured. These are morphologically indistinguishable from fibroblast-like cells cultured from human embryonic lung or skin biopsy.

Most cell strains were a mixture of cell types throughout their life in culture. However, in some cases one type or the other predominated. In this series there was a tendency for the more fibroblastic cultures to live longer however, some died after less than 10 passages whereas strains in which epithelioid cells predominated lived for more than 20 passages.

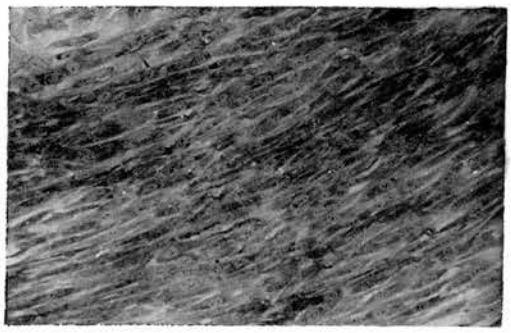

FIG. 6. Fibroblast-like cells in the fourth passage of culture. (Giemsa stain, × 200.)

The karyology of 12 of the cell strains was monitored to w ithin at least three passages before cell death. At least 10 metaphases per cell strain were examined at this stage and no departure from normal diploid complements were seen, except for that present in two of the cell strains as a constitutional abnormality.

All except five cell strains were tested for mycoplasma contamination on at least one occasion near the end of their life in culture. All cell strains tested were reported free of mycoplasmas.

Discussion

It is apparent that amniotic fluid cell strains have a highly variable yet finite life-span in culture. In the series reported here senescent strains and actively proliferating strains were in culture at the same time, receiving the same culture medium and being kept under apparently identical conditions. This would suggest that culture factors were not responsible for the variation. The longest surviving cell strain in this series survived 29 subcultures. This, however, is not the upper limit of life of an amniotic fluid cell strain; Melancon *et al* (1971) have reported that some can be cultured for more than 30 passages, and other cell strains since this series was completed have survived more than 50 passages.

The reasons for the variability in the life of the cell strains remain unclear. In all cases they are derived from fetal tissue and as the survival in culture was not correlated with gestational age, it is unlikely that the variable age of the cells which initiate the primary culture is the reason. Martin, Sprague, and Epstein (1970) have shown that the life span of fibroblast-like cells in tissue culture depends on the tissue used to establish the culture. Skin-derived fibroblasts survived longer than those derived from muscle, testis, or bone marrow spicules. Although the tissue of origin of the cells in amniotic fluid is uncertain they probably come from more than one source (Sutherland and Bain, 1972). Some of the variability seen could possibly be due to different samples of amniotic fluid having different proportions of cells from each possible source, each with a different survival potential in tissue culture.

As there are only a relatively small number of points of outgrowth in the primary culture the number of cell doublings would be much higher than the

number of passages would imply. This may well be the reason why amniotic fluid cell strains appear to have a shorter life in culture than cell strains grown from other fetal tissues. However, if this was the reason for the variation in the length of life in culture, a correlation between number of passages till death and time in primary culture would be expected, this was not found. The fact that the life in culture is finite is not surprising as Hayflick (1965) has shown that human diploid cells have a limited life span of 50 ± 10 passages when grown from fetal tissue.

The finding that the faster growing cell strains live longer is unexpected. This has not been previously reported for human diploid cell strains and the reasons for it remain unknown. Hay (1970) has in fact reported the exact opposite effect for chick embryo fibroblasts.

The variability in life-span of the amniotic fluid cell strains is very like that described by Swim and Parker (1957) for fibroblast-like cell strains. These workers found that the number of times they could subculture cell strains grown from neonatal foreskins ranged from 10 to 34 with a mean of 24. Cell strains grown from other tissues, including human embryonic lung, survived from two to 32 subcultures. They attributed this variability to inadequate tissue culture medium. Perhaps the same explanation may apply to the amniotic fluid cell strains. Using medium similar to that used in the present series but with only 15% fetal calf serum supplement, Nadler and Gerbie (1970) were able to subculture only 75% of 155 successful primary amniotic fluid cell cultures more than three times. Sutherland and Bain (1973) found that 90% of 62 primary cultures could be subcultured to yield cell strains which could be maintained for at least two subcultures.

The findings in the groups of cell strains grown from serial samples of amniotic fluid are not surprising. As none of the properties studied are related to gestation there is no reason why these cell strains should show either constant features or any trends within the groups. It would be of interest to culture a number of cell strains from a single amniotic fluid sample and to see if they showed the same degree of variation as seen in these groups.

Melancon *et al* (1971) suggested that cell type may influence length of life in culture. If cell type did affect life in culture then the number of passages till death would be expected to show a multimodal distribution. In this series the distribution of the number of passages is fairly evenly around the mean.

The variety of cell types present in amniotic fluid has received little attention. Gerbie *et al* (1972) have said that amniotic fluid cell strains are composed of two major cell types, epithelial-like cells and fibroblast-like cells. Melancon *et al* (1971) have commented that the epithelial-like cells are difficult to subculture and can be maintained for only two to five passages. It is recognized that medium composition can alter cell morphology (eg, Griffiths, 1973). In this regard it should be noted that for this series the medium was prepared in large batches and that the various cell types not only co-existed in the same culture vessel but cell strains of predominantly different morphologies were being cultured concurrently. The only comprehensive report of the various cell types is that of Uhlendorf (1970). He has described fibroblast-like cells and epithelial-like cells corresponding to types II and III of this report. The 'small fusiform cells' which he describes probably correspond to the type I epithelioid cells of this report. When the islands in which these cells grow become large, the cells in the centre of the island become crowded and take on an appearance very similar to that shown in Uhlendorf's Fig. 2. In addition, Uhlendorf described a cell type not recognized in the present series (Fig. 5 in Uhlendorf, 1970).

It is clear that amniotic fluid cell strains are composed of a number of different cell types and that some, if not all, of these can persist as long as the cell strain survives. If the various tissue culture media in general use and the variety of conditions under which these cells can be cultured had the effect of selecting against any particular cell type then this could explain the apparent differences reported in the behaviour of amniotic fluid cell strains. The very act of subculturing may in fact be such a selective mechanism as the fibroblast-like cells are more readily removed from the growth surface than epithelial-like ones. Melancon *et al* (1971) have indeed used this feature to select cell strains of different morphology.

The findings of normal karyology in the cell strains monitored until senescence is not surprising. The types of chromosome changes which have been described in senescent human diploid fibroblast-like cells (Saksela and Moorhead, 1963) would not have been demonstrated due to the small number of cells examined but any major departure from euploidy would have been detected. No cell lines or cell strains other than those derived from amniotic fluid or urine (Sutherland and Bain, 1972) were handled in the laboratory during the time this work was in progress.

The main question posed by this work is why cannot all amniotic fluid cell strains be maintained

for the same length of time as cell strains derived from other fetal tissues? One reason is probably the low cell number used to initiate cultures but this is only part of the answer. The reasons for the variability in life expectancy of these cell strains remain speculative. The factors which prevent these cultures from having a prolonged existence should be identified. The loss of valuable amniotic fluid cell strains at an early passage is a hindrance to building up adequate controls for the antenatal diagnosis of genetic disease using cultured amniotic fluid cells.

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References

Butterworth, J., Sutherland, G. R., Broadhead, D. M., and Bain, A. D. (1973). Lysosomal enzymes of cultured amniotic fluid cells. *Clinica Chimica Acta*, **44**, 295–299.

Emery, A. E. H. (1970). *Modern Trends in Human Genetics*, vol. 1. Butterworth, London.

Emery, A. E. H. (1973). *Antenatal Diagnosis of Genetic Disease*. Churchill Livingstone, Edinburgh and London.

Gerbie, A. B., Melancon, S. B., Ryan, C., and Nadler, H. L. (1972). Cultivated epithelial-like cells and fibroblasts from amniotic fluid: their relationship to enzymatic and cytologic analysis. *American Journal of Obstetrics and Gynecology*, **114**, 314–320.

Griffiths, J. B. (1973). The effects of adapting human diploid cells to grow in glutamic acid media on cell morphology, growth and metabolism. *Journal of Cell Science*, **12**, 617–629.

Hay, R. J. (1970). Cell strain senescence in vitro: cell culture anomaly or an expression of a fundamental inability of normal cells to survive and proliferate. In *Aging in Cell and Tissue Culture*, ed. by E. Holečková and V. J. Cristofalo, pp. 7–24. Plenum Press, New York.

Hayflick, L. (1965). The limited *in vitro* lifetime of human diploid cell strains. *Experimental Cell Research*, **37**, 614–636.

Littlefield, J. W. (1971). Problems in the use of cultured amniotic fluid cells for biochemical diagnoses. *Birth Defects: Original Article Series*, **7**, pt. 5, pp. 15–17. National Foundation—March of Dimes, New York.

Martin, G. M., Sprague, C. A., and Epstein, C. J. (1970). Replicative life-span of cultivated human cells. Effects of donor's age, tissue and genotype. *Laboratory Investigation*, **23**, 86–92.

Melancon, S. B., Lee, S. Y., and Nadler, H. L. (1971). Histidase activity in cultivated human amniotic fluid cells. *Science*, **173**, 627–628.

Milunsky, A. (1973). The prenatal diagnosis of hereditary disorders. Charles C. Thomas, Springfield.

Nadler, H. L. and Gerbie, A. B. (1970). Role of amniocentesis in the intrauterine detection of genetic disorders. *The New England Journal of Medicine*, **282**, 596–599.

Saksela, E., Moorhead, P. S. (1963). Aneuploidy in the degenerative phase of serial cultivation of human cell strains. *Proceedings of the National Academy of Sciences, U.S.A.*, **50**, 390–395.

Sutherland, G. R. (1972). The role of amniocentesis in genetic counselling. *Australian Journal of Mental Retardation*, **2**, 85–92.

Sutherland, G. R. and Bain, A. D. (1972). Culture of cells from the urine of newborn children. *Nature*, **239**, 231.

Sutherland, G. R. and Bain, A. D. (1973). Antenatal diagnosis of inborn errors of metabolism: Tissue culture aspects. *Humangenetik*, **20**, 251–256.

Sutherland, G. R., Brock, D. J. H., and Scrimgeour, J. B. (1973a). Amniotic fluid macrophages and anencephaly. *Lancet*, **2**. (In press.)

Sutherland, G. R., Grace, E., and Bain, A. D. (1973b). Metaphase chromosomes from neonatal urine. *Humangenetik*, **17**, 273–275.

Swim, H. E. and Parker, R. F. (1957). Culture characteristics of human fibroblasts propagated serially. *American Journal of Hygiene*, **66**, 235–243.

Uhlendorf, B. W. (1970). Use of amniotic fluid and reliability of diagnostic procedures. In *Early Diagnosis of Human Genetic Defects*, ed. by M. Harris. Fogarty International Center Proceedings No. 6, pp. 149–168. Government Printing Office, Washington.

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LYSOSOMAL ENZYME VARIATIONS IN THIRTEEN CELL STRAINS CULTURED FROM ONE AMNIOTIC FLUID

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Summary

Fifteen primary amniotic fluid cultures were established from a single sample of amniotic fluid. Three different methods were used to set up these cultures which yielded 13 cell strains. Nine lysosomal enzymes (acid phosphatase, β-glucuronidase, β-galactosidase, α-galactosidase, α-glucosidase, α-mannosidase, α-arabinosidase, *N*-acetyl-β-D-glucosaminidase and arylsulphatase A) were assayed in these 13 cell strains. The coefficients of variation of these enzyme levels were less than the coefficients for enzyme levels in cell strains grown from different samples of amniotic fluid but greater than those for the combined culture and assay system used. No assay values were found which could have suggested a possible enzyme deficiency disease.

Introduction

Lysosomal enzymes are involved in a number of inborn errors of metabolism, many of which can now be diagnosed antenatally [1]. The activities of these enzymes fluctuate in amniotic fluid cell strains [2–4] and fibroblast-like cell strains [5,6] assayed at different stages of culture. Multiple fibroblast-like cell strains grown from a single skin biopsy have been shown to have considerable variation in the activity of five lysosomal enzymes [7]. This variation was so great as to suggest that a lysosomal enzyme deficiency disease might have been wrongly diagnosed. The possibility that a similar situation may exist for cell strains cultured from a single sample of amniotic fluid has been investigated.

Materials and Methods

Amniotic fluid was collected from a pregnancy of 18 weeks gestation, terminated because of severe maternal pre-eclamptic toxaemia. The fluid was divided into 15 aliquots of 5 ml and one primary culture was established from

TABLE I

TIMES IN CULTURE FOR THE 3 METHODS OF ESTABLISHING CULTURES

Behaviour of the 15 amniotic fluid cultures (a—o) with time in primary culture and time from the first subculture until the cells in the third and sixth passages were harvested for enzyme assay.

Method of primary culture and cell strain designation		Days in primary culture	Days until 3rd passage harvest	Days until 6th passage harvest
I	a	43	—*	—
	b	—**	—	—
	c	38	46	60
	d	36	53	78
	e	36	51	82
II	f	25	37	51
	g	28	39	58
	h	25	39	58
	i	22	37	53
	j	22	37	51
III	k	22	39	56
	l	23	37	58
	m	22	39	60
	n	22	39	58
	o	22	37	58

* Cell strain died in the first passage.
** Primary culture degenerated and could not be subcultured.

The assay values of the nine lysosomal enzymes measured at the third and sixth passages are shown in Table II. To compare the variations in enzyme level in the two passages and to establish their relationship with the variations found in a series of amniotic fluid cell strains cultured from different samples of amniotic fluid [10], the coefficients of variation were calculated and are shown in Table III. For this calculation the values for the three cell strains derived from primary cultures established using Method I were excluded because of their different behaviour in tissue culture. The coefficients of variation of the combined assay and culture system, determined by assays on replicate cultures as previously described [2], are also included in Table III.

The coefficients of variation for each enzyme in the third and sixth passages are similar in magnitude for all enzymes except α-mannosidase and α-arabinosidase. The coefficients for all the enzyme levels in the third and sixth passages are smaller than those found for the control series of cell strains cultured from different samples of amniotic fluid and greater than those for the combined culture and assay system.

Discussion

Cultured amniotic fluid cells are the most reliable material for enzyme assay on which antenatal diagnoses of inborn errors of lysosomal enzyme metabolism are to be based [1,13,14]. It is thus essential that the two principle techniques involved in such diagnoses, tissue culture and enzyme assay, must combine to produce reliable and reproducible results. The establishment of a

TABLE II

SPECIFIC ACTIVITIES OF THE LYSOSOMAL ENZYMES IN THE THIRTEEN CELL STRAINS ASSAYED AT THE THIRD AND SIXTH PASSAGES OF CULTURE

Results are expressed in nmoles 4-methylumbelliferone/min per mg protein, except for arylsulphatase A which is expressed in nmoles nitrocatechol/min per mg protein.

Method of primary culture	Cell strain	Acid phosphatase	β--Glucuronidase	β--Galactosidase	α--Galactosidase	α--Glucosidase	α--Mannosidase	α--Arabinosidase	N-Acetyl-β-D--glucosaminidase	Arylsulphatase A
I	c	4.00*	0.94	5.93	0.51	0.60	1.27	0.16	35.8	1.14
		5.90**	0.87	5.66	0.59	0.45	0.83	0.19	39.1	2.37
	d	3.62	1.30	11.46	0.49	0.64	1.83	0.28	47.3	4.08
		4.98	0.64	11.27	0.58	0.64	1.09	0.25	62.3	5.40
	e	4.31	0.51	4.87	0.50	0.32	1.39	0.11	37.0	1.98
		5.97	0.24	3.72	0.36	0.21	0.65	0.10	30.7	2.78
II	f	5.01	1.40	7.89	0.57	0.47	1.00	0.21	43.2	1.82
		4.92	0.91	5.59	0.60	0.29	1.02	0.13	37.5	2.37
	g	6.18	0.86	6.88	0.60	0.43	1.11	0.21	44.7	1.67
		5.92	0.60	5.77	0.55	0.32	0.77	0.14	33.6	1.59
	h	4.88	0.64	6.48	0.52	0.39	1.11	0.20	38.0	1.72
		5.09	0.75	6.40	0.53	0.35	0.68	0.15	36.6	1.89
	i	4.18	1.41	7.28	0.53	0.53	1.00	0.22	41.2	1.90
		5.56	0.95	7.13	0.47	0.51	0.99	0.18	35.9	2.53
	j	4.61	0.96	5.92	0.69	0.29	1.09	0.15	42.6	1.80
		4.47	1.24	7.59	0.62	0.49	1.12	0.20	47.7	2.46
III	k	5.49	1.04	7.52	0.56	0.39	1.09	0.21	43.3	2.64
		6.21	1.13	6.95	0.61	0.42	0.66	0.18	43.5	2.74
	l	5.05	1.32	6.42	0.68	0.33	1.02	0.16	41.9	1.46
		6.24	0.97	6.14	0.57	0.36	0.61	0.16	39.7	2.49
	m	6.10	0.91	9.74	0.60	0.58	1.33	0.29	49.7	2.61
		7.42	0.60	6.87	0.60	0.49	0.56	0.19	44.7	2.07
	n	4.90	1.49	8.71	0.54	0.69	1.06	0.26	58.1	2.18
		5.82	0.95	6.79	0.62	0.56	0.70	0.18	44.5	1.80
	o	5.49	1.13	5.63	0.58	0.34	0.81	0.13	42.7	1.52
		5.69	0.76	5.47	0.49	0.37	0.56	0.14	34.5	1.63

* Assayed in the third passage of culture.
** Assayed in the sixth passage of culture.

TABLE III

COEFFICIENT OF VARIATION VALUES OBTAINED

Mean, standard deviation and coefficient of variation for the lysosomal enzymes in the third and sixth passages of culture and the coefficient of variation of a control series of cell strains cultured from different samples of amniotic fluid and for the combined assay and culture system. N.D. = not done.

Cell strains	Acid phosphatase	β--Glucuronidase	β--Galactosidase	α--Galactosidase	α--Glucosidase	α--Mannosidase	α--Arabinosidase	*N*-Acetyl-β-D--glucosaminidase	Arylsulphatase A
Third passage									
Mean	5.19	1.12	7.25	0.587	0.444	1.062	0.204	44.5	1.93
S.D.	0.63	0.28	1.27	0.058	0.125	0.129	0.49	5.58	0.416
Coefficient of variation	12.1	25.2	17.6	9.93	28.1	12.2	23.8	12.5	21.5
Sixth passage									
Mean	5.73	0.88	6.50	0.566	0.416	0.767	0.165	39.8	2.16
S.D.	0.82	0.21	0.70	0.054	0.091	0.203	0.024	4.95	0.413
Coefficient of variation	14.4	23.7	10.8	9.51	21.9	26.5	14.6	12.4	19.1
Control (third passage) Coefficient of variation	36.5	40.0	42.8	35.7	47.4	46.1	47.2	32.1	N.D.
Combined system Coefficient of variation	7.4	4.5	5.2	3.3	8.5	6.5	5.2	7.1	N.D.

number of primary cultures from a single large sample of amniotic fluid and enzyme assay on the cell strains subsequently produced allows both laboratory procedures to be tested simultaneously.

Much of the delay in achieving sufficient cells for a biochemical assay is taken up by the time in primary culture. Hence three methods of primary culture were tested. The first method used has been claimed to produce such rapid growth of primary cultures that sufficient cells for cytogenetic studies could be obtained in an average of seven days [8]. No other published method of primary culture has reported such rapid cell growth. The second method used has been employed to study the delay due to tissue culture in the antenatal diagnosis of inborn errors of metabolism [15]. The third method involved a modification of the culture maintenance schedule of the second, similar to that of other published methods [16,17]. The results show that the first method was inferior to the other two for this sample of amniotic fluid. In addition, the variation in the enzyme levels in the three cell strains eventually derived from the primary cultures set up using the first method was greater than that seen in the 10 strains derived from the other primary cultures.

There are two main features of this variation in enzyme levels in a series of cell strains derived from one sample of amniotic fluid. The first is that this variation is considerably less than that found in a series of cell strains derived from different amniotic fluid samples. There are several reasons for this other than the common origin of the cell strains; they were all in tissue culture at the same time, receiving the same batch of culture medium and fetal calf serum and all the enzymes were assayed at the same time. In spite of these constant conditions of tissue culture and enzyme assay the highest value of some enzymes was more than twice the lowest. Such variation would cast doubt upon heterozygote identification based on enzyme assay of a single sample of cultured cells. Some cell strains even retained widely different enzyme levels in both passages as, for example, the α-glucosidase level in strain *n* which was approximately twice that in strain *o* in both passages assayed. If the cell strains derived from cultures established by method I are considered then this variation is even more marked. Strain *d* had β-galactosidase and arylsulphatase A levels which were much greater than in the other cell strains although the rest of the enzymes in this culture were within the range for the other cell strains.

The second main feature of the results is their relationship to the only comparable study [7], in which cultured fibroblast-like cells were used. Great variations were found in the levels of several lysosomal enzymes in multiple fibroblast-like cell strains grown from a single human foreskin. For β-galactosidase, there was more than a 3-fold difference between the highest and lowest values whereas in the present study this difference was relatively small. The two series agree on the finding for *N*-acetyl-β-D-glucosaminidase which showed little variation and for β-glucuronidase in which there was a 2.5-fold difference between the highest and lowest assay values. The results of the present study for arylsulphatase A are not comparable with those quoted by Milunsky et al. [7] as their substrate and experimental conditions were not specific for arylsulphatase A [18,19].

The results of this study on cell strains derived from one sample of amniotic fluid are in agreement with previous findings [10] on cell strains derived

from different amniotic fluids. Enzyme assay of normal amniotic fluid cell strains should not produce values suggestive of an enzyme deficiency disease.

Acknowledgement

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References

1 A. Milunsky, The Prenatal Diagnosis of Hereditary Disorders, Charles C. Thomas, Springfield, 1973.
2 J. Butterworth, G.R. Sutherland, D.M. Broadhead and A.D. Bain, Life Sci., 13 (1973) 713.
3 G.R. Sutherland, J. Butterworth, D.M. Broadhead and A.D. Bain, Clin. Genet., (1974) in the press.
4 J. Butterworth, G.R. Sutherland, D.M. Broadhead and A.D. Bain, Clin. Genet., (1974) in the press.
5 W.R. Den Tandt and A. Schaberg, Pathol. Eur., 8 (1973) 3.
6 B. Hultberg, S. Sjöblad and P.A. Ockerman, Acta Paediat. Scand., 62 (1973) 474.
7 A. Milunsky, C. Spielvogel and J.N. Kanfer, Life Sci., 11 (2) (1972) 1101.
8 C. Gray, R.G. Davidson and M.M. Cohen, J. Pediat., 79 (1971) 119.
9 G.R. Sutherland, E. Grace and A.D. Bain, Humangenetik, 17 (1973) 273.
10 J. Butterworth, G.R. Sutherland, D.M. Broadhead and A.D. Bain, Clin. Chim. Acta, 44 (1973) 295.
11 G.L. Miller, Anal. Chem., 31 (1959) 964.
12 H. Baum, K.S. Dodgson and B. Spencer, Clin. Chim. Acta, 4 (1959) 453.
13 R.G. Davidson and M.C. Rattazzi, Clin. Chem., 18 (1972) 179.
14 H.L. Nadler, R.H. Bigley and G. Hug, Lancet, ii (1970) 369.
15 G.R. Sutherland and A.D. Bain, Humangenetik, 20 (1973) 251.
16 M.E. Ferguson-Smith, M.A. Ferguson-Smith, N.C. Nevin and M. Stone, Brit. Med. J., 4 (1971) 69.
17 H.L. Nadler, Pediatrics, 42 (1968) 912.
18 A.S. Perumal and E. Robins, J. Neurochem., 21 (1973) 459.
19 M.T. Porter, A.L. Fluharty, S.D. de la Flor and H. Kihara, Biochim. Biophys. Acta, 258 (1972) 769.

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Lysosomal Enzyme Levels in Human Amniotic Fluid Cells in Tissue Culture

II. α-galactosidase, β-galactosidase and α-arabinosidase

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Changes in the activities of α-galactosidase, β-galactosidase and α-arabinosidase in amniotic fluid cells with time in culture were studied. Marked fluctuations in all three enzymes occurred with passage. In certain cell strains, β-galactosidase showed a marked rise in activity correlated with passage. The activity of all three enzymes, in amniotic fluid cells at the third passage, was correlated with the total time taken to reach confluency. There was no consistent pattern of enzyme activity associated with the time after subculture. Enzyme levels in cell strains deived from serial samples of amniotic fluid from several women showed large differences in activity unrelated to gestational age.

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The number of diseases resulting from a known deficiency of a lysosomal enzyme has increased rapidly in recent years. With the demonstration that these deficiencies can be detected in cultured amniotic fluid cells, antenatal diagnosis for inborn errors of lysosomal metabolism has become practicable (Brady 1973, Nadler 1970, 1972, Prescott et al. 1972). However, little attention has been paid to possible fluctuations in the levels of lysosomal enzymes in cultured normal amniotic fluid cells (Beutler et al. 1971, Butterworth et al. 1973a, b, Gerbie et al. 1972). The effect of culture time on the activity of lysosomal enzymes in normal amniotic fluid cell strains has, therefore, been investigated. The results for α-galactosidase, β-galactosidase and α-arabinosidase are presented as part of this study.

Methods

Amniotic fluid samples were obtained from hysterotomy specimens and by amniocentesis of Rhesus iso-immunised women who had no history of inborn errors of lysosomal metabolism. Primary cell cultures were set up (Sutherland et al. 1973), then subcultured, harvested and homogenized (Butterworth et al. 1973a), and the lysosomal enzymes α-galactosidase, β-galactosidase and α-arabinosidase assayed using 4-methylumbelliferone glycosides (Butterworth et al. 1972). Protein concentration was assayed (Miller 1959) using bovine serum albumin as standard.

The reproducibility of the combined culture and enzyme assay methods was tested by establishing 12 replicate cultures of one

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cell strain at two different passages. The 12 replicates were harvested when they had simultaneously reached confluency, and then assayed for enzyme activity. To study enzyme activity with respect to passage, six cell strains were subcultured for up to 15 passages and the enzyme levels assayed at intervals. All the cell strains were assayed at the third passage, and the time interval between subculture and harvest (confluency) noted. The variation in enzyme level within a single passage was followed by establishing a series of replicate cultures, which were harvested for enzyme assay at daily intervals for up to 7 days.

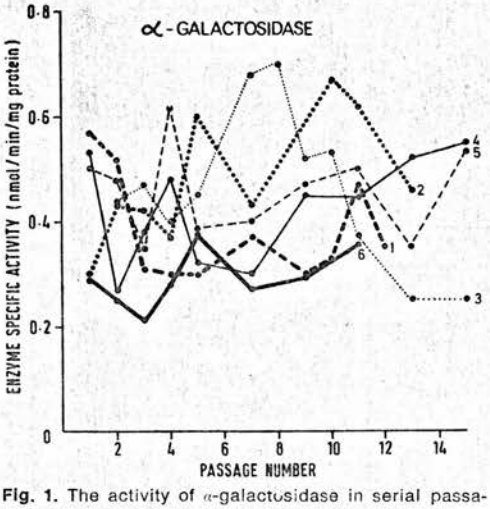

Fig. 1. The activity of α-galactosidase in serial passages of six strains of cultured amniotic fluid cells.

Results

The reproducibility of the combined culture and assay system for each enzyme is presented in Table 1. The variation of the total experimental procedure (coefficient of variation) was below 6.0% for all the enzymes. The variation in the levels of the three enzymes with passage is given for six cell strains in Figs. 1–3. The coefficient of variation of the mean enzyme levels for the different cell strains was 20–33% for α-galactosidase, 27–68% for β-galactosidase and 18–39% for α-arabinosidase. Thus, all three enzymes showed much greater fluctuations with passage than could be attributed to experimental error alone.

A comparison of the enzyme levels in the first five passages with those in the next 10 for these six cell strains showed that β-galactosidase activity was lower ($P < 0.005$) in the earlier passages. However, the mean activity of β-galactosidase in the third and tenth passages of these six strains, plus an additional six strains, was not sig-

Table 1

Reproducibility of culture and assay system

Enzyme	Mean*	s. d.	Coefficient of variation (%)
α-Galactosidase	0.90[a]	0.03	3.1
	0.40[b]	0.01	3.3
β-Galactosidase	6.11[a]	0.32	5.2
	8.58[b]	0.32	3.7
α-Arabinosidase	0.86[a]	0.05	5.2
	0.61[b]	0.02	3.3

* Enzyme activity in nmol 4-methylumbelliferone min^{-1} mg^{-1} protein for 12 replicates

a – Assay tenth passage; b– Assay twentieth passage

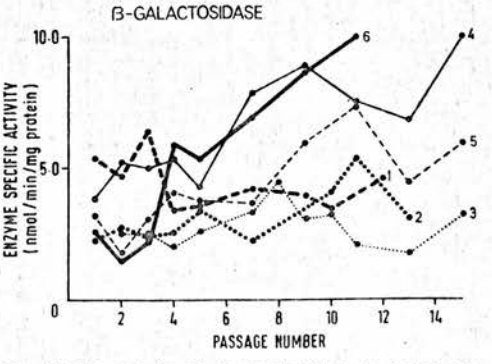

Fig. 2. The activity of β-galactosidase in serial passages of six strains of cultured amniotic fluid cells.

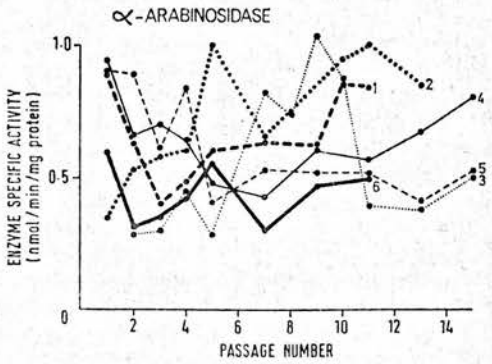

Fig. 3. The activity of α-arabinosidase in serial passages of six strains of cultured amniotic fluid cells.

nificantly different. In these 12 strains the activities of α-galactosidase and α-arabinosidase at the third and tenth passages did not differ significantly. The contradictory results for β-galactosidase could be accounted for by three of the six strains examined in detail (strains 4–6, Fig. 2), which showed a marked rise in activity with passage not apparent in the other strains.

The relationship of the enzyme activity in cell strains at the third passage to the

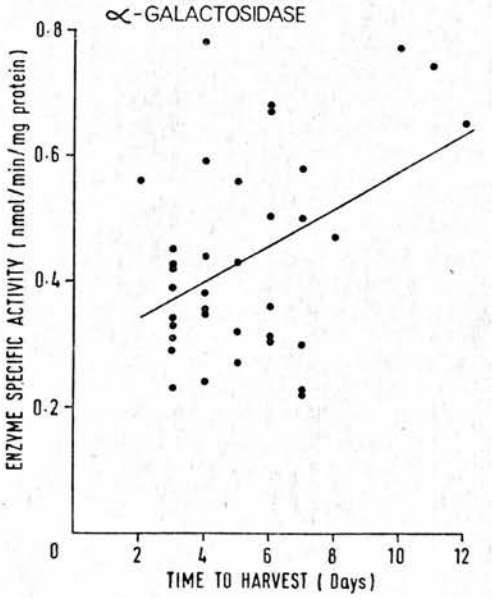

Fig. 4. Relationship of the activity of α-galactosidase in cultured amniotic fluid cells with time to reach confluency (harvest).

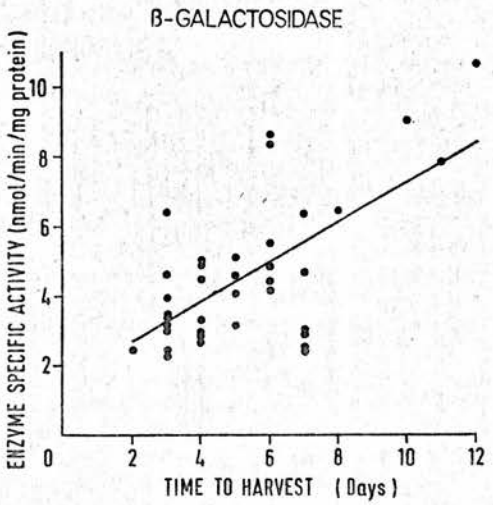

Fig. 5. Relationship of the activity of β-galactosidase in cultured amniotic fluid cells with time to reach confluency (harvest).

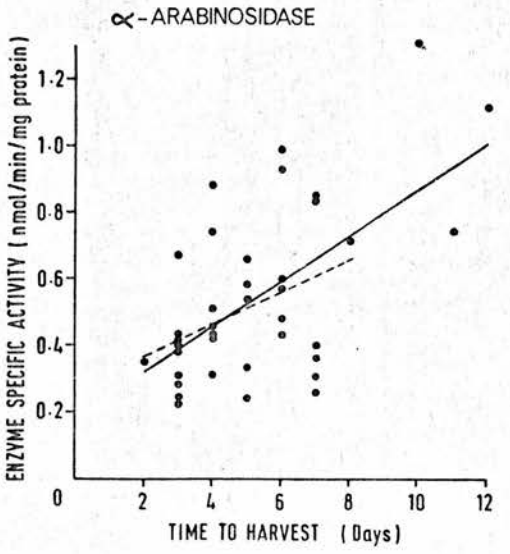

Fig. 6. Relationship of the activity of α-arabinosidase in cultured amniotic fluid cells with time to reach confluency (harvest).
——— All days - - - - - Without days 10–12

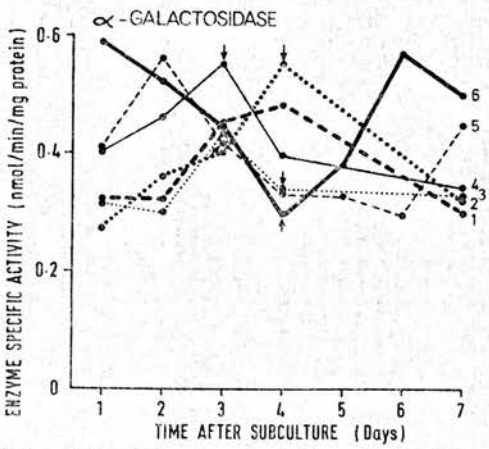

Fig. 7. The activity of α-galactosidase in six strains of amniotic fluid cells in relation to time since subculture.
♦Confluency; Strain 1 not confluent by 7 days

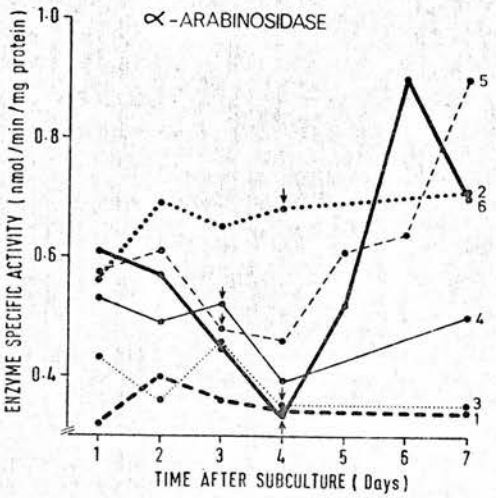

Fig. 9. The activity of α-arabinosidase in six strains of amniotic fluid cells in relation to time since subculture.
♦Confluency; Strain 1 not confluent by 7 days

time at which coufluency (harvest) was reached is given in Figs. 4–6. A significant correlation between these two parameters was found for α-galactosidase (r = 0.42), β-galactosidase (r = 0.64) and α-arabinosidase (r = 0.61). However, if the few cell strains requiring 10–12 days to reach confluency were not considered, only the correlation for α-arabinosidase (r = 0.37) was still significant.

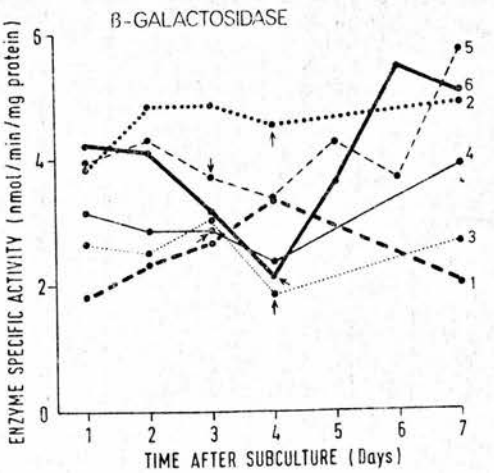

Fig. 8. The activity of β-galactosidase in six strains of amniotic fluid cells in relation to time since subculture.
♦Confluency; Strain 1 not confluent by 7 days

Table 2

Enzyme levels* in cell strains at the third passage cultured from serial samples of amniotic fluid obtained from four women.

Series	Gestational age (weeks	α-Galacto-sidase	β-Galacto-sidase	α-Arabino-sidase
1	18	0.30	2.87	0.47
	22	0.59	4.51	0.74
	24	0.78	5.01	0.88
	33	0.30	9.55	0.66
	35	0.44	5.48	0.38
2	23	0.35	2.87	0.48
	26	0.33	4.63	0.38
	28	0.29	4.96	0.22
3	22	0.65	10.76	1.11
	26	0.37	7.85	0.74
4	27	0.32	3,17	0.53
	34	0.35	2.67	0.31

* Enzyme activity – nmol 4-methylumbelliferone min^{-1} mg^{-1} protein

The fluctuations in the levels of the three enzymes in relation to time after subculture are given in Figs. 7–9. No consistent change was associated with the time after subculture. There was also no consistent change in enzyme activity after the time at which the cells became confluent.

Cell strains were grown from serial samples of amniotic fluid obtained from several Rhesus iso-immunised women and the levels of the enzymes, at the third passage, are given in Table 2. The activity of all three enzymes showed considerable variation within each series, that did not relate to gestational age.

Discussion

The activities of α-galactosidase, β-galactosidase and α-arabinosidase in normal amniotic fluid cells have been shown to vary with the time in tissue culture. The significance to the results will be considered in relation to similar studies on other lysosomal enzymes in the following paper (Butterworth et al. 1974).

References

Beutler, G. H., W. Kuhl, F. Trinidad, R. Teplitz & H. L. Nadler (1971). β-glucosidase activity in fibroblasts from homozygotes and heterozygotes for Gaucher's disease. *Amer. J. hum. Genet.* **23**, 62–66.

Brady, R. O. (1973). The abnormal biochemistry of inherited disorders of lipid metabolism. *Fed. Proc.* **32**, 1660–1667.

Butterworth, J., F. Scott, W. M. McCrae & A. D. Bain (1972). Lysosomal enzymes of cultured fibroblasts of Cystic Fibrosis patients. *Clin. chim. Acta* **40**, 139–142.

Butterworth, J., G. R. Sutherland, D. M. Broadhead & A. D. Bain (1973a). Lysosomal enzymes of cultured amniotic fluid cells. *Clin. chim. Acta* **44**, 295–299.

Butterworth, J., G. R. Sutherland, D. M. Broadhead & A. D. Bain (1973b). Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. I. α-glucosidase and β-glucosidase. *Life Sci.* **13**, 713–722.

Butterworth, J., G. R. Sutherland, D. M. Broadhead & A. D. Bain (1974). Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. III. β-glucuronidase, N-acetyl-β-D-glucosaminidase, α-mannosidase and acid phosphatase. *Clin Genet.*

Gerbie, A., S. B. Melancon, C. Ryan & H. L. Nadler (1972). Cultivated epithelial-like cells and fibroblasts from amniotic fluid: Their relationship to enzymatic and cytologic analysis. *Amer. J. Obstet. Gynec.* **114**, 314–320.

Miller, G. L. (1959). Protein determination for large numbers of samples. *Analyt. Chem.* **31**, 964.

Nadler, H. L. (1970). Newer procedures in the preconceptional, prenatal and early postnatal diagnosis of birth defects. *Birth Defects: Original Article series* VI, 26–33.

Nadler, H. L. (1972). Tissue culture and antenatal detection of molecular diseases. *Biochimie* **54**, 677–681.

Prescott, G. H., R. E. Magenis & N. R. M. Buist (1972). Amniocentesis for antenatal diagnosis. *Postgrad. Med.* **51**, 212–217.

Sutherland, G. R., E. Grace & A. D. Bain (1973). Metaphase chromosomes from neonatal urine. *Hum. Genet.* **17**, 273–275.

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Clinical Genetics 1974: **5**:

Lysosomal enzyme levels in human amniotic fluid cells in tissue culture

III, β-glucuronidase, N-acetyl-β-D-glucosaminidase, α-mannosidase and acid phosphatase

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Fluctuations in the levels of β-glucuronidase, N-acetyl-β-D-glucosaminidase, α-mannosidase and acid phosphatase in amniotic fluid cells with time in culture were studied. The four enzymes fluctuated markedly with passage; no consistent trends were apparent. The activity of α-mannosidase in amniotic fluid cell strains at the third passage was correlated with the time taken to reach confluency. There was no consistent pattern of enzyme activity associated with the time after subculture. Enzyme levels in cell strains derived from serial samples of amniotic fluid from several women showed large differences in activity unrelated to gestational age. The significance of the findings are discussed in relation to antenatal diagnosis of inborn errors of lysosomal metabolism.

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Previous reports (Butterworth et al. 1973a, Sutherland et al. 1974) have shown that the levels of five lysosomal enzymes (α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, α-arabinosidase) in amniotic fluid cells fluctuate with time in tissue culture. Similar studies have been carried out on four other lysosomal enzymes (β-glucuronidase, N-acetyl-β-D-glucosaminidase, α-mannosidase, acid phosphatase), all of which are involved in inborn errors of metabolism.

Methods

The amniotic fluid cell strains were cultured under the experimental conditions described in previous papers (Butterworth et al. 1973a, Sutherland et al. 1974). The cells were harvested and the enzymes β-glucuronidase, N-acetyl-β-D-glucosaminidase, α-mannosidase and acid phosphatase assayed using 4-methylumbelliferone conjugates as substrates (Butterworth et al. 1972, 1973b).

Results

The reproducibility of the combined culture and assay system for each enzyme is presented in Table 1. The variation of the total experimental procedure (coefficient of variation) was below 7.5% for all four enzymes. The variations in the levels of

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Table 1

Reproducibility of culture and assay system

Enzyme	Mean*	s. d.	Coefficient of variation (%)
β-Glucuronidase	2.02[a]	0.09	4.3
	0.81[b]	0.03	4.0
N-acetyl-β-D-Glucosaminidiase	87.56[a]	4.43	5.1
	41.60[b]	2.97	7.1
α-Mannosidase	0.95[a]	0.05	5.3
	1.11[b]	0.07	6.5
Acid Phosphatase	7.11[a]	0.37	5.2
	7.38[b]	0.55	7.4

* Enzyme activity in nmol 4-methylumbelliferone min^{-1} mg^{-1} protein for 12 replicates

a – assay tenth passage; b – assay twentieth passage

the four enzymes with passage is given for six cell strains in Figs. 1–4. The coefficient of variation of the mean enzyme levels for the different cell strains was 27–38% for β-glucuronidase, 16–38% for N-acetyl-β-D-glucosaminidase, 23–52% for α-mannosidase and 18–45% for acid phosphatase. All four enzymes, therefore, showed fluctuations with passage greater than could be expected from experimental error. None

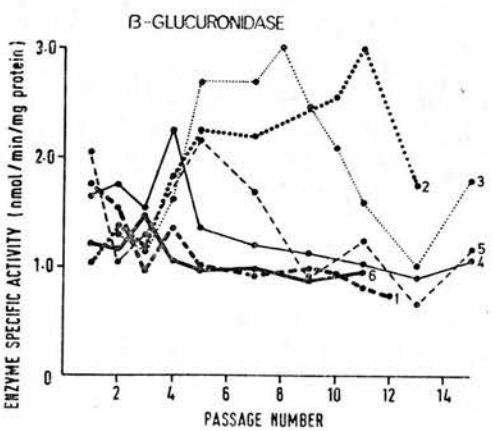

Fig. 1. The activity of β-glucuronidase in serial passages of six strains of cultured amniotic fluid cells.

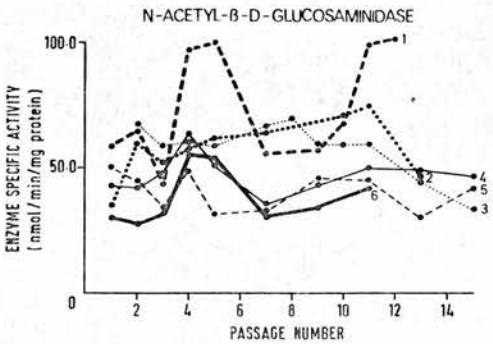

Fig. 2. The activity of N-acetyl-β-D-glucosaminidase in serial passages of six strains of cultured amniotic fluid cells.

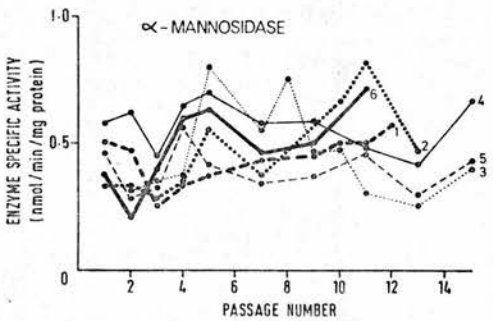

Fig. 3. The activity of α-mannosidase in serial passages of six strains of cultured amniotic fluid cells.

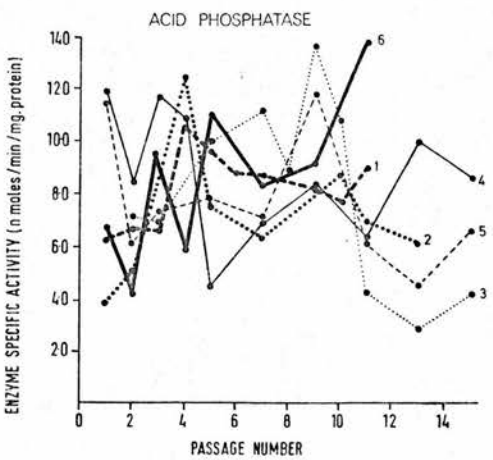

Fig. 4. The activity of acid phosphatase in serial passages of six strains of cultured amniotic fluid cells.

of these fluctuations in enzyme levels showed a consistent pattern of activity related to passage, as judged by previously described criteria (Butterworth et al. 1973a. Sutherland et al. 1974).

The results were examined to determine whether the enzyme levels of the cell strains at the third passage were related to the time at which confluency (harvest) was reached. The only significant correlation ($r = 0.58$) was for α-mannosidase (Fig. 5). This was still significant ($r = 0.42$) even if the few

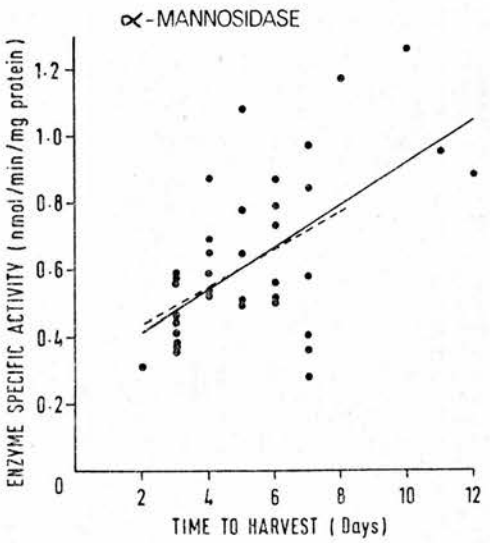

Fig. 5. Relationship of the activity of α-mannosidase in cultured amniotic fluid cells with time to reach confluency (harvest).
——— All days – – – – Without days 10–12

cell strains requiring 10–12 days to reach confluency were not included.

Figs. 6–9 show the fluctuations in the levels of the four enzymes related to the time after subculture for six amniotic fluid cell strains. No consistent pattern of enzyme activity related to this parameter was noted. Moreover, no consistent increase in enzyme activity was noted after the cell strains had become confluent.

Amniotic fluid cells were cultured from

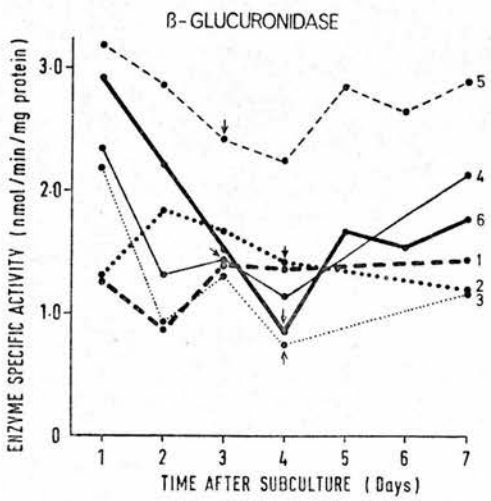

Fig. 6. The activity of β-glucuronidase in serial strains of amniotic fluid cells in relation to time since subculture.
↟Confluency; Strain 1 not confluent by 7 days

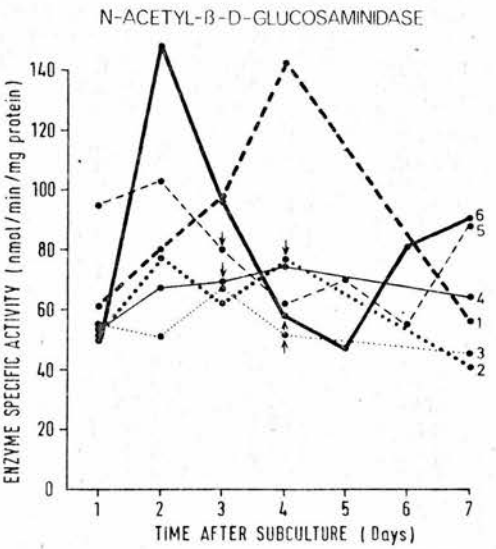

Fig. 7. The activity of N-acetyl-β-D-glucosaminidase in serial strains of amniotic fluid cells in relation to time since subculture.
↟Confluency; Strain 1 not confluent by 7 days

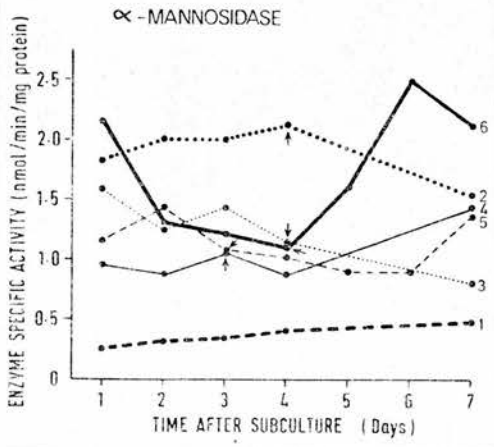

Fig. 8. The activity of ∝-mannosidase in serial strains of amniotic fluid cells in relation to time since subculture.
↑Confluency; Strain 1 not confluent by 7 days

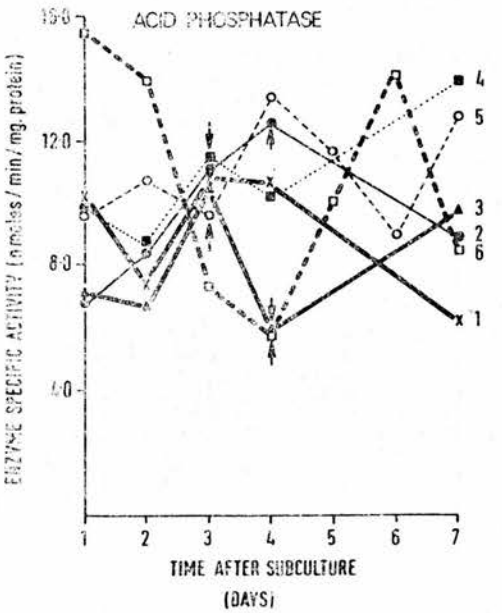

Fig. 9. The activity of acid phosphatase in serial strains of amniotic fluid cells in relation to time since subculture.
↑Confluency; Strain 1 not confluent by 7 days

serial samples of amniotic fluid obtained from four Rhesus iso-immunised women. The levels of the four enzymes in the third passage of these cell strains are given in Table 2. Within each series the activity of the four enzymes showed considerable variability, unrelated to gestational age.

Discussion

The known number of inborn errors of metabolism due to a lysosomal enzyme deficiency has increased rapidly in the last few years (Brady 1973, Milunsky 1973, Nadler 1972). Post-natal diagnosis of these diseases is made by enzyme assay using leukocytes and cultured fibroblasts. The properties and variations in activity of lysosomal enzymes in these cell types have been reported (Hultberg et al. 1973, Milunsky et al. 1972, Ryan et al. 1972).

With the demonstration that lysosomal enzymes are present in cultured amniotic fluid cells (Kaback & Cooke 1970), and that an enzyme deficiency in the fetus is reflected in these cells (Padeh & Navon 1971, Salafsky & Nadler 1971), antenatal diagnosis of inborn errors of lysosomal metabolism became possible. Indeed, there are now several reports (Brady et al. 1971, Desnick et al. 1973, Kaback et al 1973, O'Brien et al. 1971, van der Hagen et al. 1973) of the successful antenatal diagnosis of such enzyme deficiency diseases. This very success has had the result of increasing the demand for antenatal diagnosis. However, whilst there are many reports concerning lysosomal enzyme deficiencies in cultured amniotic fluid cells (Milunsky 1973), much less attention has been paid to fluctuations of these enzymes in cells cultured from normal amniotic fluids (Beutler et al. 1971, Butterworth et al. 1973a, 1973b, Gerbie et al. 1972). A study of the activities of nine lysosomal enzymes in cultured amniotic fluid cells has been

Table 2

Enzyme levels* in cell strains at the third passage cultured from serial samples of amniotic fluid obtained from four women

Series	Gestational age (weeks)	β-Glucuronidase	N-acetyl-β-D-Glucosaminidase	α-Mannosidase	Acid Phosphatase
1	18	0.60	44.4	0.28	6.35
	22	0.83	64.0	0.87	12.69
	24	0.76	57.3	0.95	11.69
	33	0.63	61.3	0.81	10.05
	35	0.75	32.2	0.42	4.41
2	23	0.43	66.5	0.52	7.15
	26	1.09	76.3	0.37	5.90
	28	0.42	34.4	0.41	11.52
3	22	0.98	91.9	0.88	9.15
	26	0.90	62.1	0.96	10.52
4	27	0.66	66.3	1.08	6.85
	34	0.64	39.4	0.59	9.67

* Enzyme activity – nmol 4-methylumbelliferone min^{-1} mg^{-1} protein

undertaken. The findings for five lysosomal enzymes have been reported (Butterworth et al. 1973a, Sutherland et al. 1974) and these will be considered together with the present results.

The levels of all the lysosomal enzymes investigated showed marked fluctuations with passage in amniotic fluid cell strains, a finding in agreement with the results of Milunsky et al. (1972) for cultured skin fibroblasts. Some amniotic fluid cell strains showed a significant pattern of increase in specific activity with passage for certain enzymes such as β-glucosidase and β-galactosidase, but only for α-glucosidase was this increase consistent. This contrasts with the general rise in lysosomal enzyme activity with passage noted in cultured fibroblasts by Hultberg et al. (1973). The ratio of epithelioid to fibroblast-like cells in amniotic fluid cell culture has been reported (Melancon et al. 1971) to change with passage. As the lysosomal enzyme levels differ between these cell types (Gerbie et al. 1972), it is possible that the patterns of change observed in the present studies reflect this change in cell type.

In cultured fibroblasts, acid phosphatase (Christofalo & Kritchevsky 1969), N-acetyl-β-D-glucosaminidase (Okada et al. 1971) and β-glucuronidase (DeMars 1964, Leroy & DeMars 1967, Russell et al. 1971) levels have been shown to be related to the time after subculture. No such relationship was consistently found for the lysosomal enzymes in cultured amniotic fluid cells. Similarly, the rise in the level of N-acetyl-β-D-glucosaminidase (Okada et al. 1971) and β-glucosidase (Ryan et al. 1972) activity in cultured fibroblasts after confluency was not apparent in cultured amniotic fluid cells. However, a positive correlation was noted between the activities of some of the enzymes in amniotic fluid cells and the time taken to reach confluency. This apparent relationship may reflect a difference between the cell types found in slowly and rapidly growing cell strains. The lack of a correlation between gestational age and the level of the lysosomal enzymes in cells

cultured from serial samples of amniotic fluid is consistent with previous findings using only one sample from any pregnancy (Butterworth et al. 1973b).

Hence, cells cultured from amniotic fluid of any gestational age will provide suitable control material for antenatal diagnosis of a disease involving any of these lysosomal enzymes. Similarly, cells at any stage of culture up to at least the fifteenth passage can be used as control material. One possible exception is α-glucosidase, where cells in later passages may have higher enzyme levels. The possibility still remains, however, that an error in diagnosis could arise due to a change in the isoenzyme pattern with gestational age or time in culture. These possibilities clearly need to be taken into consideration when making an antenatal diagnosis of a disease where only one isoenzyme is deficient.

The marked variations in the activity of the nine lysosomal enzymes described in this series of studies clearly have implications with regard to antenatal diagnosis of inborn errors of lysosomal metabolism. The fluctuations of enzyme activity in amniotic fluid cell strains with culture time, and even in cells cultured from serial samples of amniotic fluid from the same woman, result in a wide range of normal values which would make identification of heterozygotes extremely difficult. When undertaking antenatal diagnosis of recessively inherited diseases, half the fetuses encountered will be heterozygotes. If the variation in enzyme activity is as large for these heterozygotes as for the normals, then in those diseases in which the enzyme deficiency is only partial, the heterozygote could well be mistaken for an affected homozygote.

References

Beutler, E., W. Kuhl, F. Trinidad, R. Teplitz & H. L. Nadler (1971). β-glucosidase in fibroblasts from homozygotes and heterozygotes for Gaucher's disease. *Amer. J. hum. Genet.* **23**, 62–66.

Brady, R. O., B. W. Uhlendorf & C. B. Jacobson (1971). Fabry's disease: antenatal detection. *Science* **172**, 174–175.

Brady, R. O. (1973). The abnormal biochemistry of inherited disorders of lipid metabolism. *Fed. Proc.* **32**, 1660–1667.

Butterworth, J., F. Scott, W. M. McCrae & A. D. Bain (1972). Lysosomal enzymes of cultured fibroblasts of Cystic Fibrosis patients. *Clin. chim. Acta* **40**, 139–142.

Butterworth, J., G. R. Sutherland, D. M. Broadhead & A. D. Bain (1973a). Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. I. α-glucosidase and β-glucosidase. *Life Sci.* **13**, 713–722.

Butterworth, J., G. R. Sutherland, D. M. Broadhead & A. D. Bain (1973b). Lysosomal enzymes of cultured amniotic fluid cells. *Clin. chim. Acta* **44**, 295–299.

Cristofalo, V. J. & D. Kritchevsky (1969). Enzyme activity during the growth and aging of human cells. *Progr. immunobiol. Standard.* **3**, 99–105.

DeMars, R. (1964). Some studies of enzymes in cultured human cells. *Nato. Cancer Inst. Monogr.* **13**, 181–195.

Desnick, R. J., W Krivit & H. L. Sharp (1973). In utero diagnosis of Sandhoff's disease. *Biochem. Biophys. Res. Commun.* **51**, 20–26.

Gerbie, A. B., S. B. Melancon, C. Ryan & H. L. Nadler (1972). Cultivated epithelial-like cells end fibroblasts from amniotic fluid: Their relationship to enzymatic and cytologic analysis. *Amer. J. Obstet. Gynec.* **114**, 314–320.

Hultberg, B., S. Sjobland & P. A. Ockerman (1973). Properties of five acid hydrolases in human skin fibroblast cultures. *Acta paediat. scand.* **62**, 474–480.

Kaback, M. M. & R. E. Cooke (1970). Intrauterine diagnosis of fetal disorders. *Paediat. jap.* **18**, 79–87.

Kaback, M. M., H. R. Sloan, M. Sonneborn, R. M. Herndon & A. K. Percy (1973). G_{m1}-gangliosidosis type 1: In utero detection and fetal manifestations. *J. Pediat.* **82**, 1037–1041.

Leroy, L. & R. DeMars (1967). Mutant enzymatic and cytological phenotypes in cultured human fibroblasts. *Science* **157**, 804–806.

Melancon, S. B., S. Y. Lee & H. L. Nadler (1971). Histidase activity in cultivated human amniotic fluid cells. *Science* **173**, 627–628.

Milunsky, A. (1973). *The Prenatal Diagnosis*

of Hereditary Disorders. Springfield, Illinois, Charles C Thomas.

Milunsky, A., C. Spielvogel & J. N. Kanfer (1972). Lysosomal enzyme variations in cultured normal skin fibroblasts. *Life Sci.* **11**, 1101–1107.

Nadler, H. L. (1972). Tissue culture and antenatal detection of molecular diseases. *Biochimie* **54**, 677–681.

O'Brien, J. S., S. Okada, D. L. Fillerup, M. L. Veath, B. Adornato, P. H. Brenner & J. G. Leroy (1971). Tay-Sachs disease: Prenatal diagnosis. *Science* **172**, 61–64.

Okada, S., M. L. Veath, J. G. Leroy & J. S. O'Brien (1971). Ganglioside G_{M2} storage disease: Hexosaminidase deficiencies in cultured fibroblasts. *Amer. J. hum. Genet.* **23**, 55–61.

Padeh, B. & B. Navon (1971). Diagnosis of Tay-Sachs disease by hexosaminidase activity of leukocytes and amniotic fluid cells. *Israel J. med. Sci.* **7**, 259–263.

Russell, S. B., J. D. Russell & J. W. Littlefield (1971). β-glucuronidase activity in fibroblasts cultured from persons with and without Cystic Fibrosis. *J. med. Genet.* **8**, 441–443.

Ryan, C. A., S. Y. Lee & H. L. Nadler (1972). Effect of culture conditions on enzyme activities in cultivated human fibroblasts. *Exp. Cell Res.* **71**, 388–392.

Salafsky, I. S. & H. L. Nadler (1971). Alpha-1,4 glucosidase activity in Pompe's disease. *J. Pediat.* **79**, 794–798.

Sutherland, G. R., J. Butterworth, D. M. Broadhead & A. D. Bain (1974). Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. II. α-galactosidase, β-galactosidase and α-arabinosidase. *Clin. Genet.*

van der Hagen, C. B., A-L Børresen, K. Molne, G. Oftedal, K. Bjøro & K. Berg (1973). Metachromatic leukodystrophy. 1. Prenatal detection of arylsulphatase deficiency. *Clin. Genet.* **4**, 256–259.

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EFFECT OF SERUM CONCENTRATION, TYPE OF CULTURE MEDIUM AND pH ON THE LYSOSOMAL ENZYME ACTIVITY OF CULTURED HUMAN AMNIOTIC FLUID CELLS

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Summary

1. Possible changes in lysosomal enzyme activity of cultured amniotic fluid cells with serum concentration, type of medium and the pH of the medium were studied.

2. Apart from a small, but significant, decrease in the activity of β-galactosidase, none of the enzymes changed with increasing serum concentration.

3. No significant changes in enzyme activity were found between cells cultured in Ham's FlO and Eagle's MEM medium.

4. Lysosomal enzyme levels were unaffected by culturing cells for up to 9 days at pH 7.0, 7.4 and 7.9.

Introduction

As cultured amniotic fluid cells are now being used in the antenatal diagnosis of inborn errors of lysosomal enzyme metabolism, a better understanding of the reasons for the fluctuations observed in the levels of lysosomal enzymes in normal cultured amniotic fluid cells [1—4] is clearly required. It is also important to determine whether the different normal levels of enzyme activity obtained by different laboratories [5] could be attributed to variations in cell culture. Hence the effects of serum concentrations, type of tissue culture medium (Ham's FlO, Eagles's MEM) and the pH of the medium on the levels of some lysosomal enzymes in cultured normal amniotic fluid cells were investigated.

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Methods

Cell strains were initiated [6], subcultured and maintained [1] as previously described.

To study the effects of fetal calf serum concentration in the culture medium on lysosomal enzyme activity, 3 lots of culture medium (Ham's F10) were prepared with fetal calf serum concentrations of 15, 20 and 30%. The Ham's F10 (Flow Lab. Ltd) and fetal calf serum (Biocult Labs. Ltd) came from single batches: this condition was adhered to for all the culture experiments. A cell strain which had been maintained in medium with a 30% fetal calf serum concentration was used to seed 12 replicate culture bottles. 4 cultures were maintained at each serum concentration until harvested at confluency for enzyme assay. This procedure was carried out for 3 cell strains.

The effect of using two commercially available culture media, Ham's F10 (Flow Labs. Ltd) and Eagle's MEM (Wellcome Ltd) on the enzyme levels was compared. Both media were supplemented with 15% fetal calf serum. 6 cell strains which had been maintained in Ham's F10 were used for this experiment. Duplicate cultures which had been maintained in these two media for two passages were harvested at confluency for enzyme assay.

The effect of the pH of the medium on the enzyme levels was studied using Ham's F10 culture medium supplemented with 15% fetal calf serum and buffered with tricine [7]. Solutions of M tricine were prepared and the pH adjusted with 6 *N* NaOH so that when added to medium to give a final concentration of 50 mM, pH values of 7.0, 7.4 and 7.9 were obtained. In an initial experiment 12 replicate cultures were grown to confluency in Ham's F10 with 15% fetal calf serum and a bicarbonate buffer system. At this point 4 bottles were maintained in medium buffered at each of the three pH values and the cells harvested for enzyme assay 24 h later. Since this initial experiment indicated that pH did not affect the enzyme levels, another experiment was designed to determine whether growing the cells in media at different pH values from the time of seeding would affect the enzyme levels. 15 replicate cultures were prepared and medium at each pH was added to 5 bottles. To minimize pH changes the culture medium was changed every day. One culture at each pH was harvested for enzyme assay on days 1, 3, 5, 7 and 9 after the cultures had been set up. The experiment was carried out on two cell strains simultaneously, one an amniotic fluid derived fibroblast-like cell strain and the other, a similarly derived cell strain of mixed epithelioid type II and III cells [8].

The preparation of cell homogenates, protein estimation and the assay of the lysosomal enzymes were carried out as previously described [9].

Results

The levels of the lysosomal enzymes in three strains of amniotic fluid cells cultured with 15, 20 and 30% fetal calf serum supplement in the medium are given in Table I. Analysis of variance of the results indicated that there was a small, but significant, decrease in the activity of β-galactosidase ($P < 0.05$) from 15 to 30% serum levels. None of the other comparisons of enzyme activity were significantly different. There was a significantly higher level of protein ($P < 0.05$) for cell strains cultured in 30% compared to 15% serum.

TABLE I

LYSOSOMAL ENZYME ACTIVITIES OF THREE AMNIOTIC FLUID CELL STRAINS CULTURED USING DIFFERENT CONCENTRATIONS OF FETAL CALF SERUM

Figures represent the means of 4 experiments; standard deviations in parentheses.

	Enzyme activity (nmoles/min/mg protein); fetal calf serum concentration (%)								
	15	20	30	15	20	30	15	20	30
Acid phosphatase	8.06 (0.43)	7.37 (0.42)	8.90 (0.92)	5.45 (0.35)	5.94 (0.29)	5.18 (0.66)	9.62 (1.40)	9.71 (1.23)	11.19 (1.12)
N-Acetyl-β-D-glucosaminidase	36.2 (1.9)	36.6 (1.5)	33.4 (4.3)	36.5 (0.7)	33.2 (2.6)	31.7 (2.6)	88.9 (8.4)	91.0 (11.0)	101.7 (11.8)
β-Galactosidase	6.42 (0.84)	6.27 (0.74)	5.94 (0.86)	4.51 (0.16)	4.19 (0.32)	3.70 (0.34)	10.96 (1.26)	10.38 (0.88)	10.36 (0.60)
β-Glucuronidase	0.52 (0.01)	0.50 (0.04)	0.55 (0.05)	0.76 (0.06)	0.68 (0.04)	0.62 (0.05)	0.75 (0.17)	1.00 (0.22)	1.29 (0.12)
α-Galactosidase	0.27 (0.01)	0.27 (0.02)	0.28 (0.04)	0.28 (0.02)	0.28 (0.01)	0.27 (0.03)	0.55 (0.06)	0.55 (0.02)	0.50 (0.06)
α-Arabinosidase	0.24 (0.01)	0.23 (0.01)	0.20 (0.02)	0.18 (0.01)	0.16 (0.01)	0.14 (0.01)	0.34 (0.04)	0.34 (0.04)	0.34 (0.03)
α-Glucosidase	0.44 (0.03)	0.40 (0.04)	0.35 (0.04)	0.70 (0.04)	0.63 (0.06)	0.56 (0.06)	1.71 (0.11)	1.49 (0.15)	1.46 (0.16)
β-Glucosidase	0.07 (0.02)	0.08 (0.02)	0.06 (0.01)	0.06 (0.01)	0.07 (0.02)	0.06 (0.02)	0.27 (0.06)	0.27 (0.07)	0.34 (0.06)
Arylsulphatase A	0.98 (0.07)	1.11 (0.11)	1.09 (0.16)	1.07 (0.17)	0.98 (0.05)	0.84 (0.11)		Not done	
Protein (mg/ml)	0.30 (0.02)	0.33 (0.02)	0.38 (0.05)	0.24 (0.02)	0.24 (0.02)	0.28 (0.03)	0.07 (0.01)	0.09 (0.02)	0.14 (0.02)

TABLE II

LYSOSOMAL ENZYME ACTIVITIES OF AMNIOTIC FLUID CELLS CULTURED USING HAM'S F10 AND EAGLE'S MEM MEDIUM

Figures represent the means of 12 experiments; standard deviations in parentheses.

Enzyme	Enzyme activity (nmoles/min/mg protein)	
	Ham's F10	Eagle's MEM
Acid phosphatase	10.34 (3.92)	12.11 (3.64)
N-Acetyl-β-D-glucosaminidase	34.38 (7.69)	42.56 (10.02)
β-Galactosidase	6.02 (2.05)	6.41 (2.22)
β-Glucuronidase	0.89 (0.39)	0.93 (0.40)
α-Galactosidase	0.41 (0.12)	0.45 (0.14)
α-Arabinosidase	0.12 (0.06)	0.10 (0.06)
α-Mannosidase	1.21 (0.40)	1.36 (0.60)
α-Glucosidase	0.78 (0.34)	0.92 (0.25)
Arylsulphatase A	2.38 (0.73)	2.70 (1.08)
Protein (mg/ml)	0.083 (0.009)	0.059 (0.013)

The levels of the lysosomal enzymes in amniotic fluid cells cultured in Ham's F10 and Eagle's MEM medium are given in Table II. Whilst some of the enzymes in certain of the cell strains showed differences between the two media, a comparison of the results (t-test) indicated that there were no significant differences for any of the enzymes. However, the level of protein in cell strains cultured in Ham's F10 was significantly higher ($P < 0.05$) than when using Eagle's MEM.

The initial experiment on the effects of pH showed that there were no significant differences between the lysosomal enzyme activities of amniotic fluid cells when monolayer cultures were maintained at three different pH values (7.0; 7.4; 7.9) for 24 h. The results of the second experiment in which cell strains were cultured for up to 9 days at these three pH values are given in Figs. 1—3. The pattern of activity with time in culture of some of the enzymes, such as α-mannosidase (Fig. 2) and arylsulphatase A (Fig. 3), was different for the two cell strains. Inspection of Figs. 1—3 indicated that for only β-glucuronidase was there an apparent effect of pH, in that the enzyme activity at pH 7.9 was lower than that at 7.4 and 7.0 after 5 days of culture. However, a comparison (analysis of variance) of the mean enzyme activities throughout the period of culture indicated that there was no significant difference between the enzyme levels at the three pH values.

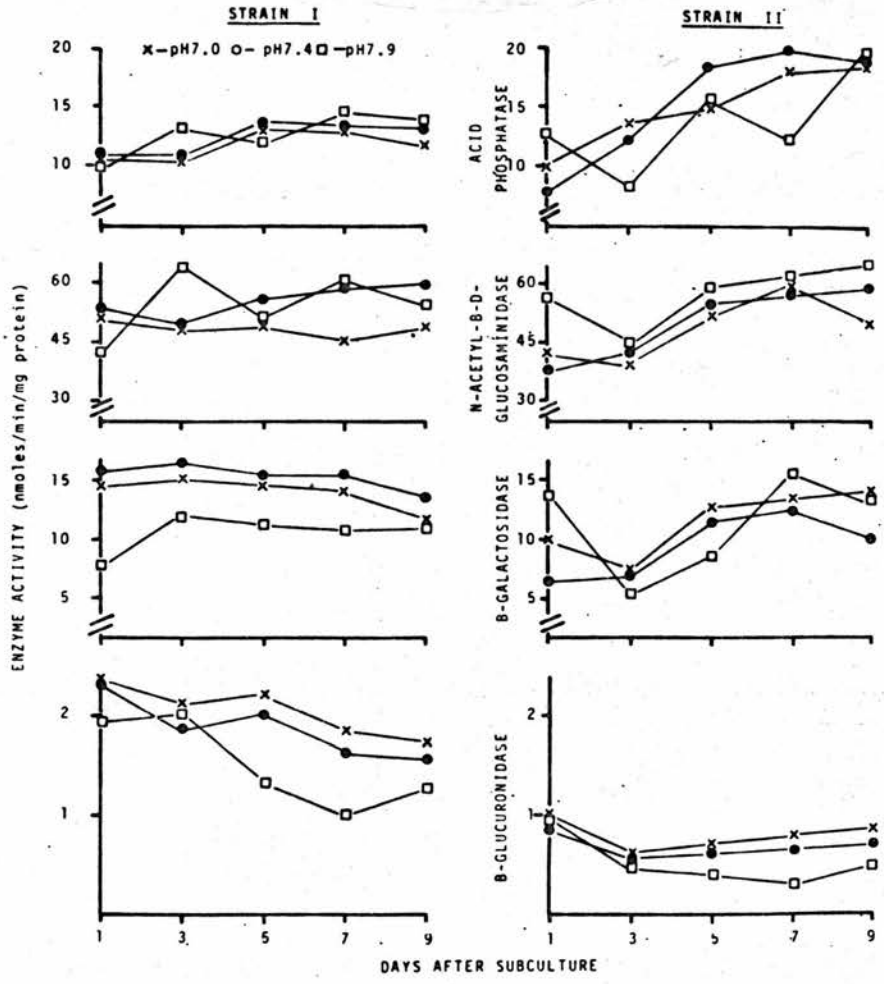

Fig. 1. The levels of acid phosphatase, *N*-acetyl-β-D-glucosaminidase, β-galactosidase and β-glucuronidase in amniotic fluid cells cultured for 9 days in medium at three pH values.

Discussion

Apart from the effects of culture time [2—4], the study of variations in lysosomal enzyme activity of amniotic fluid cells with culture variables has been neglected. The effect of serum concentration on cell growth and enzyme activity has been largely confined to cell lines requiring very low levels of serum and few studies have been undertaken using high concentrations [10,11]. Growth of rat fibroblasts was shown [10] to increase as the serum was increased from 5 to 30% with a concomitant increase in mucopolysaccharide production. A change in serum from 10 to 20% had no effect on the growth rate of human fibroblasts, but an increase to 30% led to a reduction in cell growth [11]. In the present study cell growth, as judged by protein levels, increased with serum concentration from 15 to 30% although enzyme specific

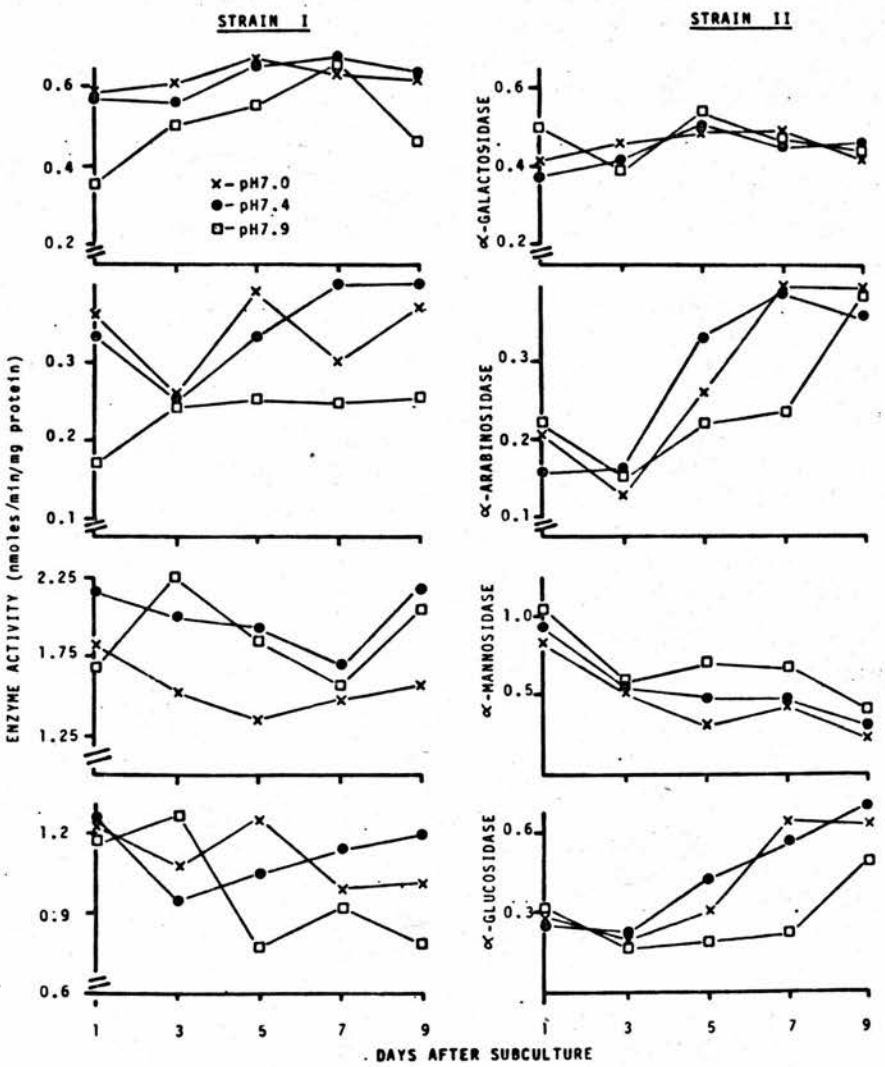

Fig. 2. The levels of α-galactosidase, α-arabinosidase, α-mannosidase and α-glucosidase in amniotic fluid cells cultured for 9 days in medium at three pH values.

activity remained constant. A possible explanation for this is that the cell strains used were adapted to growing in 30% serum. Attempts to increase the serum concentration to 30% once amniotic fluid cells were adapted to growing in 15% were unsuccessful, due to marked reduction in cell growth. In view of these effects of serum concentration on cell growth and metabolism, it is perhaps surprising that the lysosomal enzymes of amniotic fluid cells are unaffected; the statistically significant finding for β-galactosidase is probably fortuitous and of little biological significance. The finding [12] that the level of acid phosphatase in mouse fibroblasts remained unaltered when the serum concentration was changed from 0 to 10% is in accord with the present study.

The effect of using different types of media on enzyme activity has re-

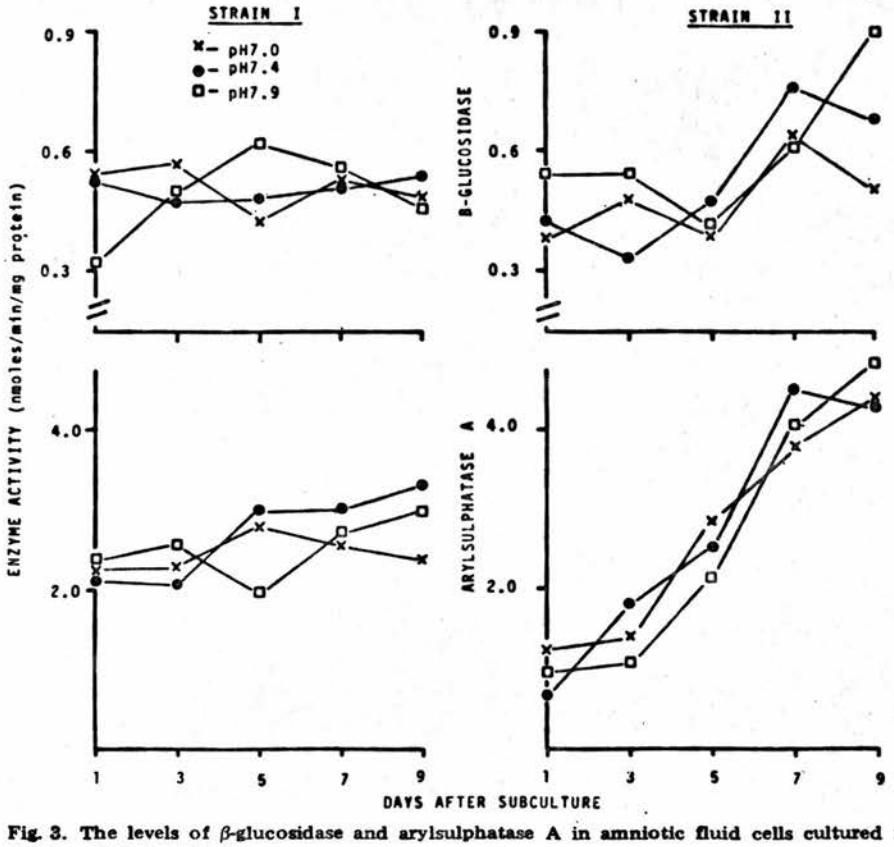

Fig. 3. The levels of β-glucosidase and arylsulphatase A in amniotic fluid cells cultured for 9 days in medium at three pH values.

ceived little attention [5,13,14]. The specific activity of lactate dehydrogenase and glucose-6-phosphate dehydrogenase has been shown [13] to be affected by the type of medium in which Earle's L cells were cultured. In cultured human fibroblasts the level of β-glucosidase was lower in cells grown in Ham's F10 than Eagle's MEM [5,14]. In the present study on amniotic fluid cells the level of acid phosphatase was found to be unaffected by these two media, in agreement with a finding for cultured human fibroblasts [14]. Although the level of arylsulphatase A has been found to be lower in fibroblasts [14] cultured in Ham's F10, no such affect was observed in amniotic fluid cells. The higher protein levels for amniotic fluid cells grown in Ham's F10 may reflect a genuine difference in the ability of these two media to support amniotic fluid cell culture , but it is more likely to be a consequence of having used cell strains adapted to growing in Ham's F10.

Although the effect of the pH of the medium on lysosomal enzyme activity has been little investigated, its affects on other aspects of metabolism have been studied. Collagen synthesis by human fibroblasts was found [15] to be greater at pH 7.4 and 8.0 than at 7.0. The effect of pH on mucopolysaccharide metabolism is perhaps of more relevance to studies involving lysosomal enzymes. The production of mucopolysaccharides by rat fibroblasts was found

[16] to be greater at pH 6.6 than pH 7.4. It was also shown [16,17] that [^{35}S]sulphate was incorporated differently into the various mucupolysaccharides at these pH values. In contrast, human fibroblast mucopolysaccharide content was found to increase with a change in the pH of the medium from 6.65 to 7.9 and this appeared to be due to a difference in degradation rather than synthesis [18]. This resulted in a great increase in intracellular membrane-bound bodies producing an abnormal ultrastructure in cells growing at their optimal pH. As acid phosphatase, a lysosomal marker, was shown [19] histochemically to be increased in activity at the higher pH there seems to be a paradox of increased lysosomal enzyme activity and at the same time decreased mucopolysaccharide degradation. In the present study biochemical assay of lysosomal enzyme levels did not reveal any difference between the activity in amniotic fluid cells cultured at about pH 7.8 as compared to lower pH values. However, the demonstration of enzyme activity in a cell homogenate or by histochemical techniques does not necessarily prove that the enzyme is functional in the living cell. It should be borne in mind that the changes in growth and metabolism of cells in a normal culture system which occur as the pH of the medium falls, may be the result of the accumulation of a metabolite, such as lactate [20], rather than to a pH change as such.

The lack of any changes in lysosomal enzyme activity of amniotic fluid cells due to the serum concentration, type of medium (Ham's F10, Eagle's MEM) and the pH of the medium indicates that these factors are unrelated to previously observed [2-4] fluctuations in these enzymes during amniotic fluid cell culture.

References

1 J. Butterworth, G.R. Sutherland, D.M. Broadhead and A.D. Bain, Clin. Chim. Acta, 44 (1973) 295
2 J. Butterworth, G.R. Sutherland, D.M. Broadhead and A.D. Bain, Life Sci., 13 (1973) 713
3 G.R. Sutherland, J. Butterworth, D.M. Broadhead and A.D. Bain, Clin. Genet., in the press
4 J. Butterworth, G.R. Sutherland, D.M. Broadhead and A.D. Bain, Clin. Genet., in the press
5 E. Beutler, W. Kuhl, F. Trinidad, R. Teplitz and H. Nadler, Am. J. Hum. Genet., 23 (1971) 62
6 G.R. Sutherland, E. Grace and A.D. Bain, Humangenetik, 17 (1973) 273
7 R. Gardner, J. Cell. Biol., 42 (1969) 320
8 G.R. Sutherland, R. Bauld and A.D. Bain, J. Med. Genet., in the press
9 G.R. Sutherland, J. Butterworth, D.M. Broadhead and A.D. Bain, Clin. Chim. Acta, 52 (1974) 211
10 P.-D. von Kittlick, G. Neupert and V. Lumkemann, Exp. Pathol., 8 (1973) 194
11 J.C. Houck and R.F. Cheng, J. Cell. Physiol., 81 (1973) 257
12 D.C. Fish and J.P. Dobbs, In Vitro, 6 (1971) 441
13 C. de Luca, Expl. Cell Res., 43 (1966) 39
14 C.A. Ryan, S.Y. Lee and H.L. Nadler, Exp. Cell Res., 71 (1972) 388
15 T.P. Nigra, G.R. Martin and H. Eagle, Biochem. Biophys. Res. Commun., 53 (1973) 272
16 P.-D. von Kittlick, G. Neupert and K.-J. Stiller, Exp. Pathol., 7 (1972) 109
17 P.-D. von Kittlick and G. Neupert, Exp. Pathol., 7 (1972) 117
18 S.O. Lie, V.A. McKusick and E.F. Neufeld, Proc. Natl. Acad. Sci., U.S., 69 (1972) 2361
19 S.O. Lie, B.H. Schofield, H.A. Taylor and S.B. Doty, Pediat. Res., 7 (1973) 13
20 P.-D. von Kittlick and G. Neupert, Exp. Pathol., 7 (1972) 125